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HIF1α is a regulator of hematopoietic progenitor and stem cell development in hypoxic sites of the mouse embryo☆

Parisa Imanirada,1, Parham Solaimani Kartalaei,1, Mihaela Crisana,1, Chris Vink,1 Tomoko Yamada-Inagawa,1 Emma de Pater,1, Dorota Kureka,1 Polynikis Kaimakis,1, Reiner van der Linden,1 Nancy Speck,2, Elaine Dzierzak1,*

a Erasmus MC Stem Cell Institute, Department of Cell Biology, Erasmus Medical Center, Rotterdam, The Netherlands
b Department of Cell and Developmental Biology, Abramson Family Cancer Research Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Abstract  Hypoxia affects many physiologic processes during early stages of mammalian ontogeny, particularly placental and vascular development. In the adult, the hypoxic bone marrow microenvironment plays a role in regulating hematopoietic stem cell (HSC) function. HSCs are generated from the major vasculature of the embryo, but whether the hypoxic response affects the generation of these HSCs is as yet unknown. Here we examined whether Hypoxia Inducible Factor1-alpha (HIF1α), a key modulator of the response to hypoxia, is essential for HSC development. We found hypoxic cells in embryonic tissues that generate and expand hematopoietic cells (aorta, placenta and fetal liver), and specifically aortic endothelial and hematopoietic cluster cells. A Cre/loxP conditional knockout (cKO) approach was taken to delete HIF1α in Vascular Endothelial-Cadherin expressing endothelial cells, the precursors to definitive hematopoietic cells. Functional assays show that HSC and hematopoietic progenitor cells (HPCs) are significantly reduced in cKO aorta and placenta. Moreover, decreases in phenotypic aortic hematopoietic cluster cells in cKO embryos indicate that HIF1α is necessary for generation and/or expansion of HPCs and HSCs. cKO adult BM HSCs are also affected under transplantation conditions. Thus, HIF1α is a regulator of HSC generation and function beginning at the earliest embryonic stages.

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Introduction

Mammalian development and many aspects of it, such as placentation, vascular development and hematopoiesis take place in a hypoxic microenvironment (Dunwoodie, 2009; Mohyeldin et al., 2010; Semenza, 2012; Simon and Keith, 2008). In the adult, hematopoietic stem cells (HSCs) localize to the bone marrow (BM) which is estimated to have oxygen levels as low as 1% (Chow et al., 2001). Several studies indicate that
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long-term repopulating HSCs are maintained at the lowest end of the oxygen gradient in the BM (Kubota et al., 2008; Parmar et al., 2007). Analysis of adult BM by the intracellular incorporation of the hypoxia marker Pimonidazole shows that quiescent HSCs are enriched in the highly hypoxic cell fraction (Takubo et al., 2010). Also, ex vivo cultures have been shown to maintain and expand repopulating HSC activity under hypoxic conditions (Danet et al., 2003). Thus, the hypoxic response is thought to protect these important stem cells from oxidative stress.

The master regulators of the hypoxic response are Hypoxia Inducible Factors (HIFs). HIFs are heterodimeric transcription factors consisting of HIF-α (HIF1α, HIF2α, and HIF3α) and HIF-β subunits (Dunwoodie, 2009; Mohyeldin et al., 2010; Semenza, 2012; Simon and Keith, 2008). HIF-β protein is constitutively present, whereas HIF-α and HIF-2α proteins are regulated by cellular oxygen concentration. Under normoxic conditions (>5% oxygen), HIF-α proteins are targeted for proteasomal degradation. In situations of hypoxia, the HIF-α proteins are stabilized in the cytoplasm, dimerize to HIF-α and translocate to the nucleus where they bind to hypoxia-responsive elements (HREs) in the promoter regions of almost 200 hypoxia-targeted genes to regulate their transcription. Most transcriptional responses to hypoxia have been attributed to HIF-1α and HIF-2α. They not only regulate the expression of many common genes such as Vegf and genes of the glycolytic pathway, but also regulate some unique target genes (Keith et al., 2012; Raval et al., 2005). HIF-1α is widely expressed in vivo and HIF-2α is also expressed in a variety of cell types (Wiesener et al., 2003).

Studies in the mouse embryo revealed central roles for HIFs in development. From embryonic day (E)8.5 onwards to E18, stabilized HIF-1α protein is detectable in the mouse conceptus (Iyer et al., 1998), confirming that many regions of the growing embryo are hypoxic (Ryan et al., 1998). Germline deletion of HIF1α (KO) results in E10.5 embryonic lethality, with a failure in placenta development, abnormal neural fold formation, defective heart and yolk sac vascular development and a smaller dorsal aorta (Cowden Dahl et al., 2005; Iyer et al., 1998; Kotch et al., 1999; Ryan et al., 1998). E9.5 KO embryos show hematopoietic defects: Erythroid progenitor numbers are reduced, BFU-E colonies are not fully hemoglobinized and the levels of Epo, EpoR, Vegf and VegFR1 mRNA are significantly decreased (Yoon et al., 2006).

Similarly, HIF2α and HIF1β germline KO embryos suffer from early embryonic lethality and show some overlapping multi-organ defects, including vascular and hematopoietic defects. Yolk sac hematopoietic progenitor activity is decreased and hematopoietic cells become apoptotic by E10.5 (Adelman et al., 1999; Maltepe et al., 1997; Ramirez-Bergeron et al., 2006). The vasculogenesis defect observed in E8.5 HIF1β KO embryos could be rescued in culture by addition of VEGF protein (Ramirez-Bergeron et al., 2006), suggesting that HIFs regulate the development of vascular/hematopoietic system. This early lethality precludes the study of HSC development. However, the role of HIF1α in the regulation of adult BM HSC function was investigated using a conditional knockout approach using Mx1-Cre:HIF1αfl/fl mice (Takubo et al., 2010). Absence of HIF1α was associated with increased cycling, leading to HSC senescence and exhaustion in serial transplantations.

The first HSCs are generated in the major vasculature (aorta–gonad–mesonephros (AGM), vitelline and umbilical arteries) of the mouse embryo at E10.5 (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996). At this time hematopoietic progenitor cells (HPCs) and HSCs emerge from vascular endothelial cells (Vascular Endothelial–Cadherin expressing; VEC+) (Chen et al., 2009; Zovein et al., 2008) in a process called endothelial-to-hematopoietic transition (EHT) (Boisset et al., 2010) and form hematopoietic cell clusters that line the arterial walls. Since HIF1α conditional deletion in adults affects HSCs, we tested whether conditional deletion of HIF1α in VEC+ cells would influence HSC generation and/or function. We show here in a VEC-Cre: HIF1αfl/fl mouse model that HIF1α regulates HPC and HSC production in the AGM and placenta at midgestation.

Materials and methods

Mice strains, embryo generation and cell preparation

HIF1αfl/fl (Ryan et al., 1998) (Jackson Laboratories) and VEC-Cre mice (Chen et al., 2009) were maintained on a C57BL/6 background. To obtain VEC-Cre/+; HIF1αfl/fl animals, VEC-Cre/+ mice were crossed to HIF1αfl/fl mice and the resulting VEC-Cre/+; HIF1αfl/fl offspring were crossed to HIF1αfl/fl mice. Genotypes were determined by PCR. Embryo production used the day of vaginal plug discovery as embryonic day 0. Somite pairs were used to stage embryos. All animal procedures were carried out in compliance with Standards for Care and Use of Laboratory Animals.

AGM, YS, PL (fetal), and FL were dissected (Robin and Dzierzak, 2005), collagenase (0.125%) treated for 45–60 min at 37 °C, washed in PBS/FCPS/PS (phosphate-buffered saline (Gibco Inc.), 10% heat-inactivated fetal calf serum (GibcoBRL), penicillin/streptomycin) and pipetted to disperse cell clumps. FL dissociation was performed only by vigorous pipetting. PL and BM cells were Ficoll fractionated. Viable cells were counted by trypan blue exclusion and kept in PBS/FCPS/PS at 4 °C. Peripheral blood erythrocytes were lysed (BD Biosciences).

PCR analysis

Embryos were genotyped with the KAPA mouse genotyping kit (KAPA Biosystems). PCR was performed with 1 μl DNA in 1× KAPA2G Fast Genotyping Mix and 0.50 μM primers: 3 min denaturation at 95 °C, 35 cycles of denaturation (15 s at 95 °C), annealing (15 s at 65 °C) [WT and fl HIF1α alleles] or 58 °C [Yav-/VEC-Cre]) and elongation (10 s at 72 °C). HIF1α primers TGTCATCAGTGCACTCT and GTGGGAGCGTAC TGGAAAG yield PCR products of 600 bp (WT) and 650 bp (fl). VEC- (Cdh5) or Yav-Cre forward primers CCCAGGCTGA CCAGCTGAG and GGCCAGCTAACGCTAACAGAAAGGG respectively and common reverse primer GCCTGGCGATCCCTGAACATG yield 300 bp or 500 bp products. HIF1α primers TGGGGATGAAACATCCTGCT and TGATGCTTCGGAGACCTG were used to detect WT (960 bp), fl (1000 bp) and recombined (Δ; 300 bp) alleles: 5 min denaturation at 94 °C, 35 cycles of denaturation (1 m at 94 °C), annealing (30 s at 59.5 °C) and elongation (15 s at 72 °C), and a final elongation (10 m at 72 °C). Ym1 primers CTGGACGTCTAC GTGATGA and CAGTTACAAATCAGCAGTCAC and myogenin primers TTAGCCTCACGTGGAACAGC and TGGGCTGGGTGT TGGCTTTA yield products of 342 bp and 245 bp respectively: 5 min denaturation at 94 °C, 29 cycles of denaturation.
was co-transplanted with 2 × 10^5 spleen cells (recipient
Two embryo equivalents (ee) of AGM cells or 1 ee of PL cells
was assessed by either Ly5.1/5.2 FACS or semiquantitative
irradiated (split dose of 9 Gy
Female, Ly5.1 or C57BL/6 recipient mice (8
In vivo
DNA isolation and recombination PCR.
CFU-C colonies were harvested in 0.5 ml lysis buffer for
microscope.

Hematopoietic cell assays

In vitro progenitor assay
Cells were plated at various dilutions in triplicate in
methylcellulose (MethoCult GF m3434, Stem Cell Technolo-
ies Inc.) with 1% PS and incubated at 37 °C, 5% CO2 for
12 days. Hematopoietic colony types were distinguished by
morphology and counted with an inverted microscope.

HSC transplantation
2 × 10^6 spleen cells or 1 ee of PL cells was co-transplanted with 2 × 10^5 spleen cells (recipient background) to support short-term survival. 2 × 10^6 spleen and 2 × 10^6 Ficoll-fractionated BM cells or 600 LSK cells from primary recipients were injected into secondary recipients.

In vivo HSC transplantations
Female, Ly5.1 or C57BL/6 recipient mice (8–20 wks) were
irradiated (split dose of 9 Gy γ-irradiation, 2.5 h apart) and
cells injected intravenously via the tail vein. Donor chimera
was assessed by either Ly5.1/5.2 FACS or semiquantitative
PCR on recipient tissues at ~4 months post-transplantation.

Flow cytometry analysis and sorting

Cells were incubated for 30 min at 4 °C with anti-CD31-PE-
Cy7, c-Kit-APC, CD41-PE, Mac1-FITC, Gr1-PE, CD8-PE,
c-Kit-PE, Sca1-FITC, B220-PE, and TER119-FITC antibodies and
Cy7, c-Kit-APC, CD41-PE, Mac1-FITC, Gr1-PE, CD4-PE, CD8-PE,
Cells were incubated for 30 m at 4 °C with anti-CD31-PE-

Hypoxyprobe labeling and whole mount embryo staining

C57BL/6 WT pregnant mice were injected with Hypoxyprobe-1 (60 mg/kg; Hypoxyprobe, Inc. Burlington, USA) and sacrificed 45 min later. E10 and E11 embryos were fixed in 4% PFA/PBS (4 °C, overnight), washed twice with PBS, paraffin-embedded and sectioned (7 μm). After deparaffination, sections were boiled (15 min) in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked (10 min with 1.5% H2O2/1% BSA/0.05% Tween/PBS). Primary antibody Hypoxyprobe-1 MAb1 (mouse 1:50; Hypoxyprobe, Inc. Burlington, USA) and HRP-conjugated secondary antibody (1:500, DAKO) were used with DAB peroxidase substrate. Sections were hematoxylin counterstained and observed with an Olympus BX40 light microscope.

As described previously (Yokomizo and Dzierzak, 2010; Yokomizo et al., 2012), whole embryos were fixed for 20–30 min in 2% paraformaldehyde/PBS on ice, and dehydrated in 100% methanol. After dissection, embryos were rehydrated, blocked (Vector Laboratories) and stained with biotinylated rat anti-mouse CD31 (MEC13.3, BD Biosciences) and subsequently rat anti-mouse c-Kit (2B8, BD Biosciences) in blocking buffer (PBS/0.4% Triton-100/1% skim milk) overnight at 4 °C and washed. Secondary antibodies were streptavidin-Cy3 (Jackson ImmunoResearch) and goat anti-rat IgG-Alexa647 (Invitrogen), respectively. Samples were analyzed on a Zeiss LSM 510 Meta microscope.

Cell cycle analysis

BM cells were stained with anti-lineage-PE antibody cocktail. Lin− cells were sorted and stained with anti-c-Kit-APC and anti-Sca1-PE-Cy7 antibodies. Cells were fixed with 2% paraformaldehyde for 1 h, washed, and incubated overnight in 0.2% Triton. After washing, cells were incubated with anti-Ki67-FITC antibody for 2 h, washed, stained with Hoechst 33342 and analyzed. IgG-FITC was used for the negative control (BD Pharmingen).

Results

HIF1α-deficiency in VEC-expressing cells affects hematopoietic progenitors in embryonic sites showing in vivo hypoxia

To examine whether hematopoietic tissues of the midgestation mouse embryo are hypoxic, pregnant dams were injected with Pimonidazole and embryos (E10 and E11) were examined for Hypoxyprobe staining. Hypoxic cells were observed in the AGM, placenta and fetal liver (Fig. 1). No background staining was found in controls, except for a few cells in the placenta. The background observed in the endodermal cell layer of the yolk sac (due to high levels of peroxidases) prevents proper evaluation of hypoxia in this tissue. The E10 AGM region showed localized hypoxic aortic endothelial cells (arrow) and hypoxic cells in emerging aortic hematopoietic cells and clusters (arrowheads). Not all hematopoietic clusters or cluster cells were hypoxic. Quantitation of hypoxic cells was performed by counting hematopoietic clusters, cluster cells and endothelial cells on transverse embryo (33μp) sections (every 5th section was counted from the forelimb to the hindlimb region): 57 ± 16% of hematopoietic clusters and 40 ± 19% of cells within the clusters were Hypoxyprobe positive. Only 19 ± 13% of endothelial cells were Hypoxyprobe positive. The mesonephric tubules were also hypoxic and in the fetal part of the E10 placenta, we found areas of high level Hypoxyprobe staining that are most likely endothelial cells (arrow). At E11, only very few, low intensity staining hypoxic cells were evident in the aorta and placenta (not shown).
However, the E11 fetal liver showed an abundance of hypoxic hematopoietic and endothelial cells (Fig. 1).

To test whether hematopoietic development in these tissues is affected by the response to hypoxia, conditional knock out (cKO) VEC-Cre/+:HIF1αfl/fl embryos were examined. The VEC-Cre mouse strain we used in these studies was previously shown to affect the recombination and expression of the LacZ reporter in all E10 aortic endothelial and hematopoietic cluster cells and provided evidence that up to 90% of FL cells and 100% of adult BM cells are derived from VEC expressing precursors (Chen et al., 2009). Based on these data, the overlap between VEC expressing cells and Hypoxyprobe positive aortic cells is expected to be complete, thus allowing the examination of the role of Hif1α in hypoxic aortic cells during EHT.

At E9 to E11, VEC-Cre/+:HIF1αfl/fl cKO embryos exhibit no visible vascular abnormalities, have normal circulation and show no fetal liver anemia. However, hematopoietic progenitor assays showed reductions in total colony forming unit-culture (CFU-C) numbers beginning at E9 in AGM and PL tissues (but not the YS) as compared to WT controls (Table 1, upper panel). At E10 total CFU-C numbers of cKO AGM and PL are significantly decreased (on average 3 and 2.1 fold, respectively) compared to WT controls (Fig. 2a). E10 cKO AGM CFU-MIX are decreased almost 5-fold, and the decrease in all other colony types is significant except for CFU-G (Table 1, lower panel). The numbers of CFU-M and CFU-MIX are also significantly decreased in E10 cKO PL. Only BFU-E were significantly reduced in E10 cKO YS, consistent with previously published data on germline HIF1α−/− YS (Yoon et al., 2006).

Decreases in total CFU-C numbers of cKO tissues persist at E11, although to a lesser extent. A significant 1.6-fold decrease is observed in AGM total CFU-C numbers (Fig. 2b and Table 1), with CFU-MIX and CFU-M significantly decreased as compared to WT. In the cKO FL, total CFU-C numbers are significantly decreased by 1.6-fold and a significant decrease is observed for cKO PL CFU-MIX, but not for total CFU-C numbers. In most cases, the heterozygous cKO tissues also showed reduced numbers of CFU-C, suggesting a HIF1α dosage effect.

Figure 1 Detection of hypoxic areas of E10 and E11 mouse embryos. Specific Hypoxyprobe (brown) staining shows areas of hypoxia in E10 (33sp) AGM, placenta and YS and E11 (44sp) FL sections (7 μm) of mouse embryos. The presence of hypoxic probe was detected in hematopoietic cluster cells (arrowheads) and some endothelial cells (arrow) in the AGM (A, B), placenta (D, E), FL (J, K) but not in the YS (G, H). Selected rectangles in the first column are shown at greater magnifications in the second column. Negative controls for each tissue staining are shown in the third column. Scale bar: 100 μm (A, C, G, I, J, L), 20 μm (B, E, H, K) and 200 μm (D, F). ms = mesonephros; nt = neural tube; cp = chorionic plate; mat = maternal part of placenta; bv = blood vessel; lb = liver lobe.
The percentage of hypoxic cluster cells found in the E10 aorta correlates with the percentage decrease in CFU-C in the E10 AGM region, suggesting that only a subset of progenitor generation/function is controlled by the Hif1α pathway. These results are not due to incomplete recombination. PCR analysis of over 330 individual colonies revealed that 85–100% of CFU-C excised both Hif1α alleles (Fig. 3). Moreover, cKO cells produce all colony types, and the morphology and size of the colonies were normal, suggesting that HIF1 does not affect progenitor function (proliferation or differentiation). Instead, our data suggest that the generation/expansion of HPCs in the Hif1α-deficient embryonic hematopoietic tissues is compromised in sites showing hypoxia.

Hematopoietic cells and clusters are quantitatively decreased in cKO embryos

To examine the hematopoietic cell content of HIF1α cKO embryonic tissues, flow cytometric analysis (FACS) was performed for c-Kit (expressed by all hematopoietic cluster cells), CD41 (expressed by some hematopoietic cluster cells), and CD31 (expressed by all endothelial and hematopoietic cluster cells) (Robin et al., 2011; Yokomizo and Dzierzak, 2010) (Table 2A). HIF1α deficient E10 AGM tissue was 4.7-fold (p < 0.05) and 1.4-fold decreased in the percentage of c-Kit+ cells and CD41int cells respectively, as compared to WT AGM tissues. A 3.6-fold decrease in CD31c-Kit+ cells (p < 0.005) and 3.4-fold decrease in CD31CD41int cells (p < 0.05) was found in cKO E10 PL, as compared to WT PL. At E11 a 1.3-fold decrease in PL c-Kit+ cells, but no decrease in AGM c-Kit+ cells and a slight decrease in AGM CD41+ cells were found in cKO embryos. The E10 YS showed slight, but not significant decreases in c-Kit+ and CD41+ cells. These fold-decreases in phenotypic hematopoietic cells in cKO AGM, PL and YS correspond well to the quantitative changes observed for CFU-Cs.

We next examined whether intra-aortic hematopoietic cluster formation is affected by the loss of HIF1α. Three-dimensional imaging was performed on E10 whole mount +/+: HIF-1αfl/fl and VEC-Cre+/+HIF-1αfl/fl immunostained embryos (c-Kit and CD31) (Yokomizo and Dzierzak, 2010). As observed by the pattern of CD31 expression, the cKO vasculature appeared normal. However, a 1.2-fold decrease in the number of hematopoietic clusters and a 1.6-fold decrease in the number of CD31c-Kit+ cells were found in cKO as compared to WT embryos (Table 2B). These data indicate that HPC/HSC formation from HIF1α-deficient hemogenic endothelium is compromised in sites showing hypoxia.

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**Table 1** Summary of CFU-C data for +/+:HIF-1αfl/fl and VEC-Cre+/+HIF-1αfl/fl embryos. (Upper panel) Hematopoietic progenitor assays were performed E9, E10 and E11 AGM, placenta (PL) and yolk sac (YS) cells and E11 fetal liver (FL) cells isolated from WT (+/+:HIF-1αfl/fl) and cKO (VEC-Cre+/+HIF-1αfl/fl) embryos (see Fig. 1). The number of embryos analyzed per genotype, somite pair stages, total CFU-C numbers per tissue (mean ± SD), fold changes of CFU-C numbers obtained from cKO tissues compared to WT controls, and significance of changes are presented here. Significant differences are indicated with asterisks. *: p-value < 0.05, **: p-value < 0.005. (Lower panel) Specific hematopoietic colony types found by methylcellulose progenitor assay of tissues of wild type (WT) and VEC-Cre:HIF-1αfl/fl embryos.

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<th>Day</th>
<th>Somite pair group</th>
<th>Number of WT embryos analyzed</th>
<th>Number of CFU-C total/WT tissue</th>
<th>Number of cKO embryos analyzed</th>
<th>Number of CFU-C total/cKO tissue</th>
<th>Fold decrease</th>
<th>p-Value</th>
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<td>3</td>
<td>130 ± 44</td>
<td>4</td>
<td>43.0 ± 20</td>
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<tr>
<td></td>
<td>11</td>
<td>43–47</td>
<td>3</td>
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<td>3</td>
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<td>9</td>
<td>20–27</td>
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<td>4</td>
<td>5 ± 7</td>
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<td>FL</td>
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<td>44–46</td>
<td>6</td>
<td>1956 ± 691</td>
<td>6</td>
<td>1236 ± 348</td>
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<td>1237.0 ± 348.0</td>
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impaired and/or the HIF1α-deficient HPC/HSC fails to expand normally after emergence.

**HIF1α-deficiency in VEC-expressing cells affects AGM and placenta long-term repopulating HSCs**

To test whether long-term HSC repopulating ability of VEC-Cre/+;HIF1αfl/fl hematopoietic tissues was affected, E11 AGM cells (1 or 2 embryo equivalents (ee)) or E11/E12 PL cells (1 or 0.3 ee) were injected into irradiated adult recipients and donor cell hematopoietic engraftment examined at greater than four months post-transplantation (Fig. 4a). Whereas 50% (3 out of 6) of recipients injected with WT AGM cells were high-level engrafted (90–95%), none of the 5 recipients receiving cKO AGM cells were repopulated to levels greater than 10%. Two cKO PL cells showed only low-level engraftment (1.2–7.4%). Similar percentages of mice were repopulated by cKO PL cells (4 out of 14) as compared to WT (4 out of 12). However, cKO PL cells yielded lower chimerism than WT PL cells (average 11% versus 28%, respectively) (Fig. 4b). Despite this lower level chimerism, cKO donor AGM

Figure 2  Hematopoietic progenitor numbers in HIF1α conditional deleted embryonic hematopoietic tissues. (a) Colony forming unit-culture (CFU-C) numbers per AGM, placenta (PL), and yolk sac (YS) in +/+;HIF1αfl/fl, VEC-Cre/+;HIF1αfl/+ and VEC-Cre/+;HIF1αfl/fl embryos at E10 (30–34 somite pair stage (sp)). (b) CFU-C numbers per AGM, PL, YS and FL at E11 (43–47sp). The colony numbers for each type (CFU-MIX, CFU-granulocyte, macrophage (GM), CFU-macrophage (M), CFU-granulocyte (G)) and BFU-E (Burst forming unit-erythroid) are shown as part of the total. Significant differences are indicated with asterisks (*: p-value ≤ 0.05, **: p-value ≤ 0.005). Data are the average CFU-C number per tissue derived from 4 to 7 embryos (3 separate litters) for each genotype (mean ± SD).
and PL cells were found to give multi-tissue (blood, BM, spleen, thymus, lymph node) and multi-lineage (B and T lymphocytes, macrophage, granulocyte and erythroid cell) repopulation (Figs. 4c and d, middle panel). Engraftment was by fully deleted HSCs, as shown by PCR analysis. Whereas AGM HSCs provided no hematopoietic engraftment in secondary recipients, the bone marrow from one of the cKO placenta cell engrafted recipients (64% primary donor chimerism) showed self-renewing capability and high level chimerism in three transplanted irradiated adult recipients (Fig. 4d, right panel). Thus, the deletion of \(HIF1\alpha\) during the generation of hematopoietic cells from embryonic vascular endothelial cells reduces the number and the quality of HSCs, supporting a role for \(HIF1\alpha\) in some aspects of HSC development and function.

**HIF1\alpha\ is required for BM HSC maintenance in stress conditions**

Despite the quantitative and qualitative decreases in hematopoietic progenitors and HSCs in cKO embryos, adult \(VEC-Cre/+/HIF1\alpha^{fl/fl}\) mice without overt phenotypic defects were obtained. FACS analysis of cKO peripheral blood, spleen, lymph node and thymus cells (defined by expression of Gr-1, Mac-1, B220, CD4, CD5, TER119 and c-Kit) revealed no significant differences as compared to WT cells (not shown). HPC frequency in cKO BM, as assessed by CFU-C assay was similar to the frequency in WT BM (Fig. 5a). Only a slight (not significant) decrease in hematopoietic chimera was observed with cKO cells in recipients when equal numbers of WT or cKO BM Lin- c-Kit+Sca1+ (LSK) cells or unfractionated adult spleen cells were transplanted. WT LSK cells showed on average 48% PBL chimerism, whereas the recipients of cKO LSK showed on average 30% chimerism (Fig. 5b). Recipients of WT and cKO spleen cells showed 51% and 40% PBL chimerism, respectively (Fig. 5C). At six months post-transplantation, all hematopoietic tissues (BM, spleen, LN and thymus) showed donor cell engraftment levels comparable to PBL chimerism (not shown). However, FACS analysis showed a significant 2.4- to 3.6-fold reduction (\(p < 0.05\)) in the frequency of BM LSK cells in recipients of cKO LSKs, as compared to recipients of WT LSK cells (Fig. 5d). This finding is consistent with the result reported by Takubo et al. (2010) and indicates that \(HIF1\alpha\) is essential for maintaining a normal level of LSK cells in stress conditions (i.e. transplantation).

**Discussion**

We demonstrate here for the first time that localized regions of hypoxia exist in the E10 aorta and placenta, as well as the liver at E11, and that the loss of \(HIF1\alpha\) leads to decreases in AGM HPCs and HSCs. By deleting \(HIF1\alpha\) specifically in embryonic endothelial cells, the precursors to emerging hematopoietic cluster cells, we have shown that \(HIF1\alpha\) regulates HSCs and HPCs during and after their generation. Deletion of \(HIF1\alpha\) leads to reductions in the number of HPCs, decreased frequencies of phenotypic HPC/HSCs, decreased aortic cluster numbers, and reductions in AGM and PL long-term repopulating HSCs. Adult BM \(HIF1\alpha\) deficient HSCs show a sensitivity to the stress conditions of transplantation. Taken together, the \(HIF1\alpha\) mediated response to hypoxia plays multiple roles in regulating HPCs and HSCs during development.

**The embryonic vasculature, hematopoietic clusters and HPC emergence**

The major embryonic vasculature is known to be the source of all definitive HPCs and HSCs, as shown by lineage tracing experiments with \(VEC-Cre\) induced marker recombination
(Chen et al., 2009; Zovein et al., 2008). VEC-Cre expression and activity begin at about E7.5 in blood islands of yolk sac, chorionic mesoderm, and vitelline artery. At E9.5, VEC-Cre activity is observed in the aorta and umbilical artery. As HPCs and HSCs are first detected at E8.5 and E10.5 respectively in the aorta, vitelline and umbilical arteries, the loss of HIF1α is likely to affect their generation from the subset of hemogenic endothelial cells lining these vessels.

Interestingly, Hypoxyprobe staining revealed hypoxic endothelial cells and hematopoietic clusters lining the aorta at E10 at the same developmental time when a significant decrease in CFU-C numbers in the cKO AGM was found. This decrease in CFU-C numbers correlates with the hematopoietic clusters and cluster cell numbers found in the E10 aorta. Similarly, the E10 PL and E11 FL show an abundance of hypoxic endothelial and hematopoietic cells that temporally correlates with a significant decrease in CFU-C numbers in these tissues of cKO embryos. It was more difficult to determine if the E10 YS is hypoxic due to background staining. However, a significant decrease in only BFU-E and not for total CFU-C was found in E10 cKO YS (Table 1, lower panel). Hence the correlation between tissue specific hypoxia and the decrease in HIF1α cKO CFU-C numbers strongly supports a role for the HIF1α hypoxic response in the generation and expansion of some HPCs.

HIF1α regulates only some HSCs

A role for the HIF1α hypoxic response in HSC generation is clearly emphasized by the fact that long-term repopulating HSCs are profoundly decreased/absent in the E10 AGM tissue, but less so in the E11 cKO PL. Hypoxyprobe analysis showed highly hypoxic cells in the aorta (endothelial cells and hematopoietic clusters), but not all hematopoietic cluster cells or clusters were Hypoxyprobe positive. This, together with the fact that all methylcellulose colonies analyzed by PCR had both HIF1α floxed alleles deleted, shows that some HPC are HIF1α-independent. Similarly, PCR analysis of long-term recipients of AGM and PL cells showed multilineage hematopoietic engraftment. However, only PL cells could be serially transplanted. These data indicate that some HSCs are HIF1α-independent and suggest that whereas the AGM may be profoundly sensitive to hypoxia and loss of HIF1α, other hematopoietic tissues such as the PL are less so, and may act as the source of the adult repopulating HSCs found in the adult BM.

Whole embryo confocal imaging also shows clear quantitative decreases in vascular hematopoietic clusters, suggesting that either the endothelial-to-hematopoietic transition is affected or the growth/expansion of the emerging HPCs and HSCs in the clusters is affected. Since the receptor tyrosine

Table 2 Percentages of phenotypic hematopoietic progenitor/stem cells in the tissues of +/+:HIF1αfl/fl and VEC-Cre/+;HIF1αfl/fl embryos. (A) Flow cytometric analysis performed on cells isolated from E10 (35–37sp) and E11 (<33sp) AGM (plus short segment of intrabody vitelline and umbilical arteries) and placenta (PL) and E10 (35–37sp) yolk sac (YS) tissues. The cellularity of hematopoietic tissues of HIF1α deficient embryos (AGM, PL and YS) was found to be the same as WT hematopoietic tissues (not shown). Cells were stained with antibodies specific for CD31, CD41 and c-Kit markers. The percentage marker positive cells within the viable single cell population are shown. Three to six tissues were analyzed (mean ± SD). Fold changes in percentage between WT and conditional knockout (cKO) tissues are shown and significant differences are indicated with asterisks (*p-value < 0.05; **p-value < 0.005). (B) Quantitation of the number of c-Kit positive cells and hematopoietic clusters in the aorta of whole mount imaged E10 +/+;HIF1αfl/fl (WT) and VEC-Cre/+;HIF1αfl/fl (cKO) embryos. The average number of c-Kit+ cells per cluster (3–4) remained the same in the cKO aorta. (n = 2).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>FACS marker</th>
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<th>Fold decrease</th>
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<tr>
<td></td>
<td></td>
<td>WT</td>
<td>cKO</td>
</tr>
<tr>
<td>E10 AGM</td>
<td>c-Kit</td>
<td>5.6 ± 3.0</td>
<td>1.2 ± 3.0</td>
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<td></td>
<td>CD41</td>
<td>1.0 ± 0.3</td>
<td>0.7 ± 0.2</td>
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<tr>
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<td>c-Kit</td>
<td>8.8 ± 0.8</td>
<td>9.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>CD41</td>
<td>2.5 ± 0.8</td>
<td>2.3 ± 0.4</td>
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<td>43.4 ± 0.5</td>
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<tr>
<th>Whole mount embryo</th>
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<td></td>
<td>WT (33sp)</td>
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<tr>
<td>c-Kit+ cells</td>
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</tr>
<tr>
<td>clusters</td>
<td>66</td>
</tr>
<tr>
<td>Average c-Kit+ cells/cluster</td>
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kinase Flk1 is known to be expressed on endothelial cells of the midgestation vasculature and moreover, on emerging aortic hematopoietic cluster cells (Yokomizo and Dzierzak, 2010) and VEGF is a direct target of HIF1α (Liu et al., 1995), it is possible that the activation of this signaling pathway in the embryonic vasculature (Lee et al., 2001) may influence EHT in the hypoxic embryonic endothelial cells. In addition, a recent study in Zebrafish embryos indicates that metabolism affects AGM HSC formation (Harris et al., 2013). Growth of embryos in high glucose increased HSC numbers through a HIF1α mediated response. A knockdown of HIF1α in embryos showed a decrease in runx1/cMyb positive cells. These data are in line with our findings that HIF1α is required for HSC formation in the dorsal aorta and highlight the evolutionary conservation of this pathway through vertebrate development. Future studies will examine the expression of such molecules, as well as other downstream targets of HIF1α in the context of endothelial-to-hematopoietic transition.

**Figure 4**  *In vivo* long-term hematopoietic transplantation analysis of HIF1α cKO AGM and placenta cells. Peripheral blood chimerism of adult irradiated recipients injected with wild type (WT) and VEC-Cre/+;HIF1αfl/fl (a) AGM and (b) placenta cells was assessed by FACS analysis for donor Ly5.2 hematopoietic cells at greater than 5 months post-transplantation. (a) Percentage engraftment in recipients injected with 2 ee of AGM cells from WT (open diamond) and cKO (black diamond), or 1 ee of AGM cells from WT (open circles) and cKO (black triangle) E11 (46–50sp stage) embryos (n = 2). (b) Percentage engraftment of recipients injected with 0.3 ee of E12 placenta cells from WT (open triangle) and cKO (black triangle), 1 ee of E12 cKO placenta cells (cross), or 1 ee of E11 placenta cells from WT (open circles) and cKO (black circles) embryos (n = 3). Average percentage donor cell chimerism for each transplantation group is shown next to the line. (c) Multilineage engraftment analysis of three recipients of VEC-Cre/+;HIF1αfl/fl E11 AGM and placenta (PL) cells and one WT control. Recipients were sacrificed at greater than 5 months post-injection and donor cell chimerism was assessed by FACS analysis for the donor cell Ly5.2 marker in peripheral blood leukocytes (PBL), bone marrow (BM), spleen (Spl) and lymph nodes (LN) and in specific lineages — B lymphoid (B), T lymphoid (T), granulocyte (G), macrophage (M) and erythroid (Ery). Percentages of donor cells in each tissue/lineage are shown. (d) PCR analysis to test for deletion of HIF1α in the donor-derived engrafted cells. PCR analysis was performed on peripheral blood leukocyte DNA from 3 recipients transplanted with +/+;HIF1αfl/fl and 3 recipients transplanted with VEC-Cre/+;HIF1αfl/fl E11 placenta cells (see panel B) at 8 months post-injection. WT cell engraftment (38% and 100%) is observed by the presence of the 1 kb HIF1α fl fragment. The presence of the recombined HIF1α (0.3 kb) fragment and indicate engraftment levels of 64%, 22% and 32% with cKO placenta cells. The wt HIF1α fragment (0.96 kb) is derived from recipient cells. All engraftment levels were consistent with the donor cell Ly5.2 marker analysis. (Middle panel) multilineage PCR analysis of peripheral blood leukocytes (pb), bone marrow (bm), spleen (sp), thymus (th), lymph node cells (ln), spleen T, B lymphocytes (sT, sB) and bone marrow erythroid, myeloid and lymphoid cells (bE, bM, bL) from the 22% engrafted recipient injected with VEC-Cre/+;HIF1αfl/fl E11 placenta cells at 8 months post-transplantation. The recombined HIF1α fragment but not the fl HIF1α fragment is visible in each lane. (Right panel) PCR analysis of three secondary recipients at 4 months postinjection of (3 × 106) bone marrow cells from primary engrafted (64%) recipient.
Our analysis of cKO adult mice showed that HIF1α also plays a role in regulating BM HSC function under stress conditions. Steady state hematopoiesis in adult cKO mice appeared normal. The effects of HIF1α deficiency were only revealed upon transplantation and examination of the BM of the primary recipients — BM HSC content (LSK cells) was significantly decreased. It has been suggested that quiescent HSCs are maintained in a hypoxic niche at a lower oxidative stress to prevent their differentiation and exhaustion. They utilize glycolytic metabolism instead of mitochondrial oxidative phosphorylation to meet energy demands and show HIF1α up-regulation (Simsek et al., 2010). Studies of adult MX1-Cre: HIF1αfl/fl mice revealed that HIF1α (but not HIF2α Guirarte et al., 2013) is required to maintain normal number of HSCs. Under stress condition such as serial transplantations, HIF1α deficient HSCs lose their quiescence (Takubo et al., 2010).

Secondary transplantations were performed to test the self-renewal capacity of VEC-Cre/+:HIF1αfl/fl cKO HSCs. LSK cells from primary recipients (WT and cKO LSK cells) with similar level of chimerism were injected into irradiated secondary recipients. Surprisingly, secondary recipients of cKO LSK cells showed a high chimerism levels at greater than 4 months post-transplantation (Figs. 5e and f), indicating that cKO BM HSCs self-renew. Recombination efficiency was approximately 46–93% for donor cells.

**HIF1α in adult BM HSCs in stress conditions**

Secondary transplantations were performed to test the self-renewal capacity of VEC-Cre/+:HIF1αfl/fl cKO HSCs. LSK cells from primary recipients (WT and cKO LSK cells) with similar level of chimerism were injected into irradiated secondary recipients. Surprisingly, secondary recipients of cKO LSK cells showed a high chimerism levels at greater than 4 months post-injection (Figs. 5e and f), indicating that cKO BM HSCs self-renew. Recombination efficiency was approximately 46–93% for donor cells.
VEC-Cre/+:HIF-1α/+/ mice to examine the role of HIF1α in the adult BM microenvironment.

In conclusion, HIF1α plays an essential role in HPC and HSC generation in hypoxic sites of the developing embryo, particularly in the hemogenic endothelial stage in the AGM, as well as the fetal placenta and liver (although to a lesser degree). Understanding the precise role of HIF1α and physiological weak oxygenation, or "in situ normoxia," in regulating the endothelial-to-hematopoietic transition can provide new insights into in vitro manipulation and culture conditions that may allow the efficient de novo generation and/or expansion of HSCs for research and clinical applications.

Conflict of interest

The authors declare no competing financial or personal interests. P Imanirad, P Solaimani-Kartalaei, M Crisan, C Vink, T Yamada-Inagawa, E de Pater, D Kurek and P Kaimakis performed research. R van der Linden collected data. P Imanirad, P Solaimani Kartalaei, M Crisan and E Dzierzak designed experiments, analyzed and interpreted data. N Speck contributed a new reagent. P Imanirad and E Dzierzak wrote the manuscript.

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