Hypoxia-inducible Factor-1 Deficiency Results in Dysregulated Erythropoiesis Signaling and Iron Homeostasis in Mouse Development*

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Hypoxia-inducible factor-1 (HIF-1) regulates the transcription of genes whose products play critical roles in energy metabolism, erythropoiesis, angiogenesis, and cell survival. Limited information is available concerning its function in mammalian hematopoiesis. Previous studies have demonstrated that homozgyosity for a targeted null mutation in the Hif1a gene, which encodes the hypoxia-responsive α subunit of HIF-1, causes cardiac, vascular, and neural malformations resulting in lethality by embryonic day 10.5 (E10.5). This study revealed reduced myeloid multilineage and committed erythroid progenitors in HIF-1α-deficient embryos, as well as decreased hemoglobin content in erythroid colonies from HIF-1α-deficient yolk sacs at E9.5. Disregulation of erythropoietin (Epo) signaling was evident from a significant decrease in mRNA levels of Epo receptor (EpoR) in Hif1a−/− yolk sac as well as Epo and EpoR mRNA in Hif1α−/− embryos. The erythropoietic defects in HIF-1α-deficient erythroid colonies could not be corrected by cytokines, such as vascular endothelial growth factor and Epo, but were ameliorated by Fe-SIH, a compound delivering iron into cells independently of iron transport proteins. Consistent with profound defects in iron homeostasis, Hif1α−/− yolk sac and/or embryos demonstrated aberrant mRNA levels of hepcidin, Fpn1, Irp1, and frascati. We conclude that dysregulated expression of genes encoding Epo, EpoR, and iron regulatory proteins contributes to defective erythropoiesis in Hif1α−/− yolk sacs. These results identify a novel role for HIF-1 in the regulation of iron homeostasis and reveal unexpected regulatory differences in Epo/EpoR signaling in yolk sac and embryonic erythropoiesis.

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Hypoxia-inducible factor-1 (HIF-1) regulates a variety of adaptive physiological responses to hypoxia, including glucose transport, glycolysis, angiogenesis, erythropoiesis, and iron metabolism (1–4). At the molecular level, HIF-1 activates the transcription of numerous genes, including vascular endothelial growth factor (VEGF), glucose transporter-1 (Glut1), Epo, transferrin (Tf), and transferrin receptor (Tfr). HIF-1 represents a dimeric protein consisting of HIF-1α and HIF-1β (also known as the arylhydrocarbon receptor nuclear translocator (Arnt) subunits (2, 3). Disruption of the Hif1α or Hif1β gene causes embryonic lethality in mice because of cardiac, vascular, and neural malformations (5–8). A Hif1β-deficient embryonic stem cell model revealed abnormal hematopoiesis, which could be partially rescued by VEGF (9). HIF-1β can dimerize with proteins other than HIF-1α, and thus the phenotype of Hif1β−/− in vitro differentiated embryoid bodies suggests, but does not prove, a role for HIF-1 in hematopoiesis (10).

In contrast to the constitutive expression of HIF-1β, HIF-1α protein levels are regulated in response to the cellular oxygen concentration. Under normoxic conditions, HIF-1α binds to the von Hippel-Lindau tumor suppressor protein (Vhl), which targets HIF-1α for ubiquitin-proteasome-mediated degradation (11–13). In contrast, under hypoxic conditions, HIF-1α is not degraded, increasing HIF-1α protein levels. In response to anemia and other causes of systemic hypoxia, HIF-1α transactivates Epo, Tf, and Tfr gene expression. In patients with Chuvash polycythemia, increased HIF-1α expression leads to increased sensitivity of erythroid progenitors to Epo (14).

HIF-1α-deficient embryos manifested disorganized vascularization of the yolk sac, although fully formed vessels containing red blood cells were present (7). Furthermore, nucleated red blood cells were detected in the dorsal aorta of the embryo proper at E9.75 (15). These data indicate that the absence of HIF-1α does not completely abrogate erythropoiesis.

3 The abbreviations used are: HIF-1, hypoxia-inducible factor-1; Epo, erythropoietin; EpoR, Epo receptor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; CFU, colony-forming units; PBS, phosphate-buffered saline; FBS, fetal bovine serum; IL, interleukin; BFU-E, burst-forming units-erythroid; E, embryonic days; Tfr, transferrin receptor; RT, reverse transcription; Tpo, thrombopoietin.
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In this study, we demonstrate that HIF-1α-deficient embryos have reduced numbers of erythroid progenitors and impaired terminal erythroid differentiation in the yolk sac, which differ from the defects described in HIF-1β-deficient embryonic stem cells. We show that these defects associate with dysregulated expression of genes encoding Epo, EpoR, VEGFR1, and iron regulatory proteins. The results establish a pivotal role for HIF-1 in the regulation of yolk sac erythropoiesis and iron metabolism in mouse development.

EXPERIMENTAL PROCEDURES

Mice and Genotyping—Hif1α+/− mice were previously generated by gene targeting and have been maintained on a mixed background (C57BL/6 × 129 genetic background) by brother-sister mating as described (5). DNA isolated from embryos or neonatal tail biopsies was used for genotyping by multiplex PCR. The PCR was performed using HotStarTaq master mix kit (Qiagen Inc., Chatsworth, CA) in a 12-μl reaction mixture (3.5 mM MgCl2, 200 mM of each deoxyribonucleotide triphosphate, 2.4 pm of each primer, 100 ng of genomic DNA, and 0.6 units of HotStarTaq DNA polymerase). A PCR condition included an initial heat activation step at 95 °C for 15 min, followed by 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 58 °C, 60 s extension at 72 °C, and a final 10-min extension at 72 °C. The multiplex PCR was used with the following primers: HIF-1α37, 5′-TTT CCA GTA CTG CCC CAA; HIF-1αREV, 5′-GCA AAC AAG CAA ATC ACC AAC G; PGK Pro-REV, 5′-GGG GCT GCT AAA GCG CAT GC, which generated 380- and 600-bp bands for the null and wild type alleles, respectively.

Yolk Sac Isolation—Hif1α+/− mice, aged 8 weeks or older, were used for timed mating. Noon of the day when the vaginal plug was detected was considered E0.5. The pregnant females were sacrificed at E9.5. Following embryo dissection, the yolk sac and embryo were washed separately three times in PBS and were sacrificed at E9.5. Following embryo dissection, the yolk sac cells were plated in duplicate at 2–2.4 × 10⁴ cells/ml/dish in 0.9% methylcellulose-based media (Stemcell Technologies, Vancouver, British Columbia, Canada) at 37 °C for 1 h. The cells from the yolk sac were either directly used for in vitro hematopoietic culture assays or suspended in Tri-reagent (Molecular Research Center, Cincinnati, OH) for RNA extraction. RNA, protein and genomic DNA were extracted in Tri-reagent from digested embryos.

Assays for Erythroid, CFU-Mix, and Mast Cell and GM Progenitors—Yolk sac cells were plated in duplicate at 2–2.4 × 10⁴ cells/ml/dish in 0.9% methylcellulose-based media (Stemcell Technologies) and incubated under the following conditions for definitive progenitor colony assay (conditions 1 to 3) or primitive erythroid colony assay (condition 4). Condition 1 is complete methylcellulose medium with pokeweed mitogen-stimulated murine spleen conditioned medium PWM-SCCM (StemCell Cult M3430). Condition 2 is “basic” methylcellulose medium with 15% FBS (MethoCult M3234) and a mixture of recombinant cytokines added to the media to obtain the following final concentrations: 10 ng/ml murine IL3 (Stemcell Technologies); 10 ng/ml human IL6 (Stemcell Technologies); 3 ng/ml murine granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN); 50 ng/ml murine SCF (Stemcell Technologies); and 3 units/ml human Epo (Epogen, Amgen, CA). For VEGF testing, basic methylcellulose medium with 30% FBS and the same combination of cytokines but with 2 units/ml Epo and, in addition, 5 ng/ml murine VEGF120 (R&D Systems) were added to the cultures. Condition 3 is FBS-free methylcellulose medium (M3236, MethoCult) supplemented with 30% FBS (HCC-6900, Stemcell Technologies), 10 ng/ml murine IL3, 10 ng/ml human IL6, 150 ng/ml murine SCF, 1 unit/ml human Epo, 100 ng/ml murine thrombopoietin (Tpo) (Stemcell Technologies). Condition 4 is FBS-free methylcellulose medium (M3236, MethoCult) supplemented with 10% fetal platelet derived serum (Animal Technologies, Tyler, TX), 5% protein-free hybridoma medium (PFHM-II, Invitrogen), 3 units/ml Epo, 10 ng/ml murine IL3, 10 ng/ml murine IL6, 50 ng/ml murine SCF, 3 ng/ml murine granulocyte-macrophage colony-stimulating factor ( PeproTech, Rocky Hill, NJ), as described previously (16), and 100 μl of a 1 mm solution of salicylaldehyde isonicotinoyl hydrazone saturated with iron (Fe-SIH) (17), an iron chelate that delivers iron for heme synthesis without involving the TfR/DMT1 pathway (17). 1 mm Fe-SIH was prepared as follows: 4.7 mg of synthesized siH (18) was dissolved in 150 μl of N NaOH and then diluted with 14.5 ml of PBS; the SIH solution was then saturated with iron by adding 7 ml of 5 m ferric citrate solution. Colony-forming units-erythroid (CFU-E) were counted at day 2 or 3; burst-forming units-erythroid (BFU-E) were counted at day 7 or 8, and mast cell and granulocyte/macrophage colonies were counted at days 7–10 and multipotent progenitor colonies (CFU-Mix) at days 10–12 of culture.

Quantitative Real Time RT-PCR—The mRNA levels of selected genes in the yolk sac and embryo were measured by one- or two-step real time RT-PCR on an ABI Prism 7000 sequence detection system (Applied Biosystems Inc., Foster City, CA). The primers and TaqMan MGB probes for each gene were either designed by using Primer Express software version 2.0 (Applied Biosystems), or obtained as a 20× Assays-on-Demand Gene Expression Assay mix commercially from Applied Biosystems. The sequences of primers/probes and commercial assay identifications are described in Table 1. The one-step real time RT-PCR was performed in a 20-μl reaction mixture using TaqMan one-step RT-PCR master mix reagents kit (Applied Biosystems), 900 nM of each primer, and 100 nM of the TaqMan probe. We used 5–500 ng of RNA/reaction giving the linear range of response for selected genes, and 100-fold diluted RNA for 18 S. The universal temperature cycling consisted of 30 min for reverse transcription at 48 °C, denaturation and polymerase activation at 95 °C for 10 min, followed by 45 cycles of denaturation at 92 °C for 15 s and annealing/extension/plate reading at 60 °C for 1 min. For two-step, initial total RNA (5–500 ng) was used for first strand synthesis using reverse transcriptase (Invitrogen) and random hexamer. The first strand synthesis followed the manufacturer’s protocol. One μl of cDNA was used in a 20-μl reaction mixture using the TaqMan Universal PCR master mix, No AmpErase® UNG reagent kit (Applied Biosystem), 900 nM of each primer, and 100 nM of the TaqMan probe using FAM and VIC fluorescence dyes (Applied Biosystems). The universal temperature cycling consisted of polymerase activation at 95 °C for 10 min, followed by 45 cycles of denaturation at 92 °C for 15 s and annealing/extension/plate reading at 60 °C for 1 min.
VEGFR1 and HIF2α were analyzed using Cyber Green dye. All samples were analyzed in duplicate wells. RT-PCR in the absence of reverse transcriptase was performed for each sample to rule out genomic DNA contamination. The relative quantitative expression of each gene in each sample was normalized to 18 S rRNA level. The cycle threshold (Ct) value for each of eight selected mRNAs and 18 S rRNA was determined, and RNA levels were calculated as ΔCt = Ct of target mRNA – Ct of 18 S. A lower value of ΔCt indicates a higher selected gene expression.

**Immunohistochemistry**—Dissected E9.5 embryos along with the attached yolk sac were fixed overnight in 4% paraformaldehyde/PBS, dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned at 5 μm. Sections from wild type and HIF-1α−/− embryos were mounted side by side on the same slide to allow for comparison of expression levels. Immunohistochemistry was performed as described (19). Briefly, sections were treated with 3% H2O2 in methanol for Fpn1 antigen retrieval. For Dmt1 and TfR detection, sections were boiled in 0.01 M citric acid, pH 6.0. Blocking was achieved with goat (Fpn1 and Dmt1) or horse serum (TfR). Primary antibody incubations were performed overnight at 4 °C using rabbit anti-Fpn1 at 1:200 (20), rabbit anti-Dmt1 at 1:100 (Alpha Diagnostic International), and mouse anti-TfR at 1:1000 dilution (Invitrogen). Secondary antibodies were peroxidase-conjugated with the Vectastain Elite ABC kit (Vector Laboratories), followed by signal detection with Vector NovaRED substrate (Vector Laboratories).

**Western Blot Analysis**—E9.5 embryos and yolk sacs were dissected from timed pregnant females, and a small portion of the embryo tail was recovered for genotyping. Embryos and yolk sacs were processed individually for Western blot analysis as described (21). Briefly, embryos and yolk sacs were homogenized and lysed in RIPA buffer plus Complete®, EDTA-free protease inhibitor (Roche Applied Science). Extract supernatant was collected and protein was quantified using the Bio-Rad DC protein assay kit (Bio-Rad). Seven and five μg of total protein from embryo and yolk sac, respectively, were mixed with an equal volume sample buffer with β-mercaptoethanol, boiled, separated on 8% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Blocking was achieved by incubation in Tris-buffered saline containing 5% milk and 0.1% Tween 20. Membranes were incubated overnight at 4 °C using the following primary antibodies: mouse anti-TfR at 1:2000 (Invitrogen) and goat anti-actin at 1:5000 dilution (Santa Cruz Biotechnology). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution), and the signal was developed using ECL reagent (Santa Cruz Biotechnology).

**Statistical Analysis**—Student’s t test and analysis of variance were used for statistical analysis. Significant differences were
considered when \( p < 0.05 \). The software Vassarstats was used for calculation.

**RESULTS**

_Hif1α-deficient Yolk Sacs Contain Blood Islands_—_Hif1α-deficient_ embryos die between E10 and E10.5 (5, 7, 15). We dissected yolk sacs at E9.5 for analysis of primitive erythroid progenitors or for analyses of definitive erythroid and CFU-Mix progenitors, respectively. At E9.5, somite counts ranged from 11 to 17 for _Hif1α−/−_ embryos to 21–25 in wild type litters. This indicated a moderate developmental delay. Furthermore, it is noteworthy that the experiments described below assess intrinsic properties of erythropoietic cells in yolk sac, which are less likely to be affected by a developmental delay of the embryo.

The yolk sacs of _Hif1α-deficient_ embryos at E9.5 were smaller (Fig. 1A) and had a significantly lower number of cells

**TABLE 2**

_Hematopoietic progenitors evaluation_

| Condition |  |  |  |  |
|-----------|---|---|---|---|
|            | _/+_ | _−/−_ | % control |
| CFU-E      | 27  | 11  | 41 |
| BFU-E      | 19  | 3   | 16 |
| CFU-Mix    | 39  | 14  | 36 |
| Mast + GM  | 56  | 25  | 45 |

| Condition |  |  |  |  |
|-----------|---|---|---|---|
|            |  |  |  |  |
| CFU-E      | 31 | 12 | 39 |
| BFU-E      | 32 | 9  | 20 |
| CFU-Mix    | 50 | 30 | 52 |
| Mast + GM  | 95 | 53 | 56 |

|            |  |  |  |  |
| Condition  |  |  |  |  |
|            |  |  |  |  |
| CFU-E      | 46 | 17 | 37 |
| BFU-E      | 151| 67 | 44 |
| CFU-Mix    | 143| 106| 74 |

_recovered after collagenase digestion compared with the wild type yolk sacs (3.6 \( \times 10^4 \) cells versus 6.1 \( \times 10^4 \) cells, respectively, \( n = 16, p = 0.002 \)) but had unambiguous blood islands (Fig. 1), whereas _Hif1α+/−_ embryos were phenotypically indistinguishable from wild type embryos (data not shown).

_Erythropoietic Defects in Hif1α−/− Yolk Sac_—To directly examine the role of HIF-1 in erythropoietic development, we studied the growth of yolk sac erythroid progenitors _in vitro_. First, we analyzed definitive erythropoiesis in the yolk sacs of _Hif1α−/−_ and wild type mouse embryos dissected from the uterus at E9.5. Yolk sac erythroid progenitor cells were analyzed by _in vitro_ hematopoietic colony assays using various conditions.

Using methylcellulose media with PWM-SCCM (see condition 1 under “Experimental Procedures”), the numbers of erythroid and CFU-Mix colonies derived from the mutant yolk sac were significantly reduced (Table 2). We considered two possible explanations for these results as follows: 1) the culture condition for progenitors from _Hif1α−/−_ yolk sacs could not support colony growth or 2) erythropoiesis in _Hif1α−/−_ yolk sac was severely impaired. To test these hypotheses, we cultured the yolk sac cells under condition 2, which favors BFU-E and CFU-Mix. Under this condition, the number of CFU-Mix from _Hif1α−/−_ yolk sacs was improved, but not the number of BFU-E and CFU-Mix (Table 2). However, the size (cellularity) of the _Hif1α−/−_ CFU-Mix colonies were 2–5 times smaller than the colonies derived from the wild type embryos (Fig. 2, C and D), and erythroid cells (BFU-E colonies and erythroid component of CFU-Mix) were not fully hemoglobinized (Fig. 2, A–D). These results indicate impaired erythropoiesis in _Hif1α−/−_ yolk sacs.

We then hypothesized that multipotential progenitors might be present in the _Hif1α−/−_ yolk sacs in comparable numbers with the wild type, but they may require higher doses of cytokines/growth factors to stimulate survival and/or proliferation. Because Tpo was shown to promote mixed lineage colony-forming cell growth in yolk sac cultures (22), we cultured the yolk sac cells in the presence of high serum levels and Tpo (condition 3). Again, the numbers of BFU-E and CFU-E derived from _Hif1α−/−_ yolk sacs were lower than wild type. However, the numbers of CFU-Mix from wild type and _Hif1α−/−_ yolk sacs were comparable (Table 2). Furthermore, the hemoglobinization of erythroid cells was slightly improved (Fig. 2, E and F). These data confirmed the presence of multipotential progenitors in the _Hif1α−/−_ yolk sacs and indicated that HIF-1α is essential for erythroid progenitor survival/growth/differentiation and for colony-forming capacity of multilineage myeloid progenitors. Analysis of primitive erythroid progenitors in the _Hif1α−/−_ and WT yolk sacs (E9.5) by using condition 4 revealed a significantly lower number and impaired hemoglobinization of _Hif1α−/−_ erythroid colonies (Fig. 3, A and B).
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Serum and Fe-SIH but Not VEGF Improve Hemoglobinization of Hif1α−/− Erythroid Colonies—Serum factors have been shown to support BFU-E in vitro (23). Increasing FBS supplementation up to 30% resulted in partial rescue of hemoglobinization of Hif1α−/− CFU-Mix (Fig. 2F) in the presence or absence of Tpo in the media (data not shown). We hypothesized that this effect may be promoted by serum factors involved either in iron delivery (e.g. transferrin) or in survival/differentiation. High dose cytokine supplementation (150 ng/ml SCF and 3 units/ml Epo) to the standard culture media (condition 3) did not rescue the defect in terminal differentiation of Hif1α−/− erythroid cells (data not shown). Similarly, the addition of 5 ng/ml VEGF to the standard culture media (condition 2) failed to improve either hemoglobinization or plating efficiency of erythroid colonies (data not shown), although it did improve the non-erythroid component of the CFU-Mix colonies (Fig. 2, G and H) and also increased plating efficiency of the non-erythroid CFU-GM colonies (data not shown).

Because supplementation with a high concentration of growth factors failed to improve erythropoiesis, we cultured the yolk sacs cells in the presence of Fe-SIH (17), a compound delivering iron into cells independently of the transferrin receptor/Dmt1 system. As shown in Fig. 3A, the addition of 100 μM Fe-SIH to condition 4 significantly increased the number of erythroid colonies derived from the wild type yolk sacs (211%, p = 0.027), even though there was no appreciable increase in the number of erythroid colonies from Hif1α−/−. However, there was significant rescue of the erythroid differentiation defect in Hif1α−/− as the size of erythroid colonies and the degree of hemoglobinization were markedly improved (Fig. 3B).

Differential Expression of Epo/EpoR and VEGFR1 Expression in Hif1α−/− Embryos and Yolk Sacs—To understand the molecular mechanisms of the erythropoietic defects in Hif1α−/− yolk sacs, we measured the mRNA levels of selected gene products related to erythropoiesis and hypoxia signaling by quantitative real time RT-PCR. As shown in Fig. 4, Epo, EpoR, and VEGFR1 mRNA levels were significantly reduced (3.6-, 2.1-, and 2.9-fold, respectively) in Hif1α−/− embryos, compared with stage-matched wild type controls. In addition, EpoR, but not Epo and VEGFR1 mRNA levels were significantly reduced (2.8-fold) in Hif1α−/− yolk sacs. In addition, the levels of mRNAs encoding erythroid-specific 5-aminolevulinate synthase and embryonic β-like globin (mHbY) were significantly lower in Hif1α−/− as compared with wild type embryos (11.8- and 7.9-fold, respectively), but in the yolk sac there was no appreciable expression difference between genotypes. In addition, Hif2α mRNA levels were not significantly different in the genotypes studied (Fig. 4).

Altered mRNA Expression of Iron Metabolism Genes in Hif1α−/− Embryos and Yolk Sacs—The partial rescue of the erythroid differentiation defect in Hif1α−/− yolk sacs by supplementation with Fe-SIH (Fig. 3B) suggested defects in iron metabolism as a contributing mechanism. To evaluate this hypothesis, we measured the level of mRNAs encoding proteins involved in iron metabolism by real time RT-PCR. As shown in Fig. 5A, the quantitative mRNA analyses revealed that Tfr and frascati mRNA levels were significantly lower (10.3- and 2-fold, respectively) in Hif1α−/− embryos compared with wild type embryos, whereas in the yolk sac no significant difference in mRNA expression between genotypes was detected. In contrast, increased levels of mRNAs encoding ferroportin (Fpn1) and iron regulatory protein 1 (IRP1) (1.5- and 1.6-fold, respectively) were observed in Hif1α−/− yolk sacs, and hepcidin mRNA expression was markedly up-regulated in both Hif1α−/− yolk sacs and embryos (4.3- and 5.4-fold, respectively). The expression of other genes involved in iron metabolism such as Dmt1 and iron regulatory protein 2 (IRP2) showed no statistically significant difference between genotypes.

Abnormal Expression of Iron Metabolism Proteins in Hif1α−/− Yolk Sacs and Embryos—At E9.5, expression of Fpn1, Dmt1 and Tfr was detected in yolk sacs, whereas expression in embryonic tissue was significantly lower and generally ranged near detection limits (Fig. 5B and data not shown). Fpn1 was expressed in visceral endoderm, yolk sac mesoderm, and in endothelial cells (Fig. 5B). Compared with wild type, Fpn1 expression was consistently increased in mesodermal and endothelial cells of Hif1α−/− yolk sac. All cell types in the yolk sac, including nucleated primitive hematopoietic cells, demonstrated Dmt1 expression (Fig.

FIGURE 3. Effect of Fe-SIH on erythroid colonies. A, erythroid colonies were counted at day 8 of culture. The number of erythroid colonies derived from Hif1α−/− yolk sacs was significantly lower than one from wild type yolk sac. By adding Fe-SIH, the number of erythroid colonies was significantly increased in wild type yolk sac, although one from Hif1α−/− yolk sac shows only a modest increase. B, panels a and b, erythroid colonies from wild type yolk sac treated with PBS or Fe-SIH, respectively. Panels c and d, erythroid colonies from Hif1α−/− yolk sac treated with PBS or Fe-SIH, respectively. Compared with wild type control (panel a), the erythroid colonies from Hif1α−/− yolk sac were smaller and poorly hemoglobinized (panel c). The addition of Fe-SIH improved the size and hemoglobinization of mutant erythroid colonies (panel d), approaching the aspect of wild type colonies (panel b). Original magnification, ×50. * represents p < 0.05.
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FIGURE 4. RNA analysis of selected erythroid- and hypoxia-controlled genes. mRNA levels of the genes are described as differences in the value of cycle threshold normalized to 18S mRNA level, i.e. \( \Delta C_T = C_T - C_{18S} \) of 18S. The lower value of the \( \Delta C_T \) indicates a higher gene mRNA level. 1 unit difference of \( \Delta C_T \) represents 2-fold transcript amount changed. Student’s t test was used for statistical analysis to compare Hif1α+/− with Hif1α−/− embryos or yolk sacs. *, \( p < 0.05 \); **, \( p < 0.001 \).

5B). Dmt1 expression levels were indistinguishable between wild type and Hif1α-deficient yolk sac.

Although TfR expression was readily apparent in wild type visceral endoderm, nucleated primitive hematopoietic cells as well as mesodermal and endothelial cells demonstrated markedly lower TfR protein levels (Fig. 5B). Strikingly, TfR expression was significantly decreased in visceral endodermal cells of Hif1α−/− yolk sacs compared with wild type. Furthermore, consistent with the immunohistochemistry, Western blot analysis identified significantly decreased TfR protein levels in Hif1α−/− yolk sacs as well as in the embryo proper (Fig. 5C).

DISCUSSION

Maintenance of oxygen homeostasis is critical for multiple biological processes, including mammalian embryonic development. The appropriate response to hypoxia is required for embryonic metabolism and orderly cardiovascular development (1) and possibly the establishment of hematopoiesis (9, 10). During murine embryogenesis, expression of the VEGF receptor Flk1 in yolk sac cells is essential for initiation of hematopoiesis and blood island formation (24). Furthermore, targeted disruption of VEGF revealed a dose-dependent requirement during mouse embryogenesis, because heterozygosity for the VEGF null allele caused embryonic lethality at midgestation because of impaired angiogenesis and blood island formation (25, 26). Based on transcriptional regulation of VEGF by HIF-1 (27, 28) and studies in HIF-1β-deficient mice, it was proposed that hypoxia controls the formation and function of hematopoietic and endo-
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High levels of Epo, as well as of VEGF and other cytokines, failed to rescue the defect in terminal differentiation of HIF1α−/− erythroid cells. HIF-1 directly regulates expression of the Epo gene (2), and a recent report showed that EpoR is also regulated by HIF-1 in vascular endothelial cells (32). This study showed that HIF-1 regulates EpoR expression in both the embryo and the yolk sac. Collectively, our results indicate that HIF-1α promotes yolk sac erythropoiesis by regulating at least two major hypoxia response pathways (Epo/EpoR and VEGF/VEGFR).

Interestingly, our results revealed that HIF-1 regulated Epo expression in the embryo but not in the yolk sac. These findings confirmed previous reports on the differential regulation of Epo and EpoR in yolk sac (33, 34). Conceivably, compensatory up-

white arrow; endothelial cells, white arrowhead). Low levels of Fpn1 expression in nucleated primitive hematopoietic cells (black arrowhead) could not be confirmed nor excluded. Analysis of Hif1α−/− yolk sacs revealed discrete but reproducible up-regulation of Fpn1 expression in mesodermal (white arrow) and endothelial cells (white arrowhead). By immunohistochemistry, Dmt1 expression was identified in all cell types, and there was no detectable difference between wild type and Hif1α−/− yolk sac. Visceral endoderm cells (black arrow) manifested relatively high TfR protein levels and lower expression in nucleated primitive hematopoietic cells (black arrowhead) and mesodermal cells (white arrow). Scale bar in all panels, 0.02 mm. Western blot analysis revealed decreased TfR expression in Hif1α−/− yolk sac and embryo (both represented as −/−) compared with wild type (represented as +/+). Approximate molecular masses, TfR, 95 kDa; actin, 41 kDa.
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High levels of hepcidin expression could contribute to the defects in erythropoiesis in Hif1α+/− yolk sac because hepcidin was shown to inhibit erythroid colony formation at low concentration of Epo in vitro (45). Interestingly, the erythropoietic defects described in this study are not due to a block in the developmental switch from embryonic to fetal/adult erythropoiesis because HbB1 fetal/adult globin expression was detected in Hif1α+/− erythroids and yolk sacs, whereas embryonic globin HbY mRNA levels decreased. This contrasts with a previous study (46) using embryoid bodies, which proposed a hypoxia-regulated developmental switch in globin expression.

HIF-1 plays a significant role in adult erythropoiesis, as demonstrated by the congenital up-regulation of HIF-1α in Chuvash polycythemia. This autosomal recessive disorder is caused by homozygosity for the VHL R200W mutation in the von Hippel-Lindau gene (14), and derives, at least in part, from an increase in serum Epo levels. The erythroid progenitors also displayed increased sensitivity to Epo under in vitro conditions, implicating erythropoiesis promoting factor(s) yet to be identified (47). In conjunction with our previous studies on Chuvash polycythemia (14), the differential response of erythropoietic cells of Hif1α+/− and Hif1α−/− yolk sacs to Fe-SIH-mediated cellular iron delivery implicates an unknown HIF-1α-responsive, iron-containing factor in the promotion of erythropoiesis.

In conclusion, deficiency for HIF-1α differentially affects Epo/EpoR signaling during the embryo and yolk sac development. This study also revealed that HIF-1α plays a critical role in stimulating the survival, proliferation, and differentiation of erythroid progenitors in yolk sac. Finally, HIF-1 regulates TfR expression in the yolk sac and embryo proper, thereby providing iron for erythropoiesis during early embryonic development.

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