Hypoxia-Inducible Factor 1alpha (HIF1A) Mediates Distinct Steps of Rat Trophoblast Differentiation in Gradient Oxygen¹

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ABSTRACT

Defective differentiation of invasive, placental trophoblast cells has been associated with several pregnancy-related disorders. This study examines the molecular, functional, and morphological differentiation of lineage-specific, trophoblast giant cells under a gradient of oxygen concentrations. Low oxygen (3%) inhibited differentiation, but this inhibition was relieved in a stepwise fashion with increasing levels of oxygen. The oxygen-sensitive hypoxia-inducible factor 1alpha (HIF1A) is a major transcriptional regulator of the cellular response to low oxygen, and increased HIF1A protein levels and activity corresponded with the maintenance of the stem cell-like state and inhibition of trophoblast differentiation in low oxygen. Furthermore, constitutive expression of an oxygen-insensitive, active form of HIF1A protein mimicked the effects of low oxygen, inhibiting the differentiation of trophoblast giant cells. This study is the first to delineate the stepwise effects of oxygen on the activation of the trophoblast giant cell differentiation process and establishes a new paradigm from which to investigate trophoblast differentiation. In addition, this is the first reported study to demonstrate that constitutive HIF1A activity mediates oxygen's inhibition of differentiation. These results suggest that a dysregulation of HIF1A could contribute to impaired placental development.

differentiation, gradient oxygen, Hif1a, hypoxia, hypoxia-inducible factor, placenta, trophoblast

INTRODUCTION

The placenta is the definitive feature of eutherian mammals and a unique organ that is transiently formed during pregnancy to fulfill a multitude of functions for the developing fetus, including attachment, nutrient and waste exchange, endocrine secretion, and respiration. Gross defects in placental development can result in spontaneous abortion or early embryonic lethality. Thus, understanding the processes that mediate the establishment and function of the placenta are important in determining the causes for miscarriage, intrauterine growth

retardation, and other pathologies associated with improper placental development, such as pre-eclampsia [1–12].

In rodents, at least two different stages of trophoblast giant cell development can be defined: an early stage of endovascular invasion that occurs through midgestation, when trophoblast giant cells associate with the maternal arteries and displace endothelial cells, and a later period of interstitial trophoblast invasion occurring only after midgestation, when trophoblasts invade the decidual stroma, but do not appear to be associated with maternal blood vessels [13]. Models for studying placental development include the Rcho-1 rat trophoblast cell line, a well-established model system used to investigate trophoblast differentiation [14–21]. Rcho-1 cells are an excellent model for examining the differentiation process, as they are a reported placental stem cell-like model committed to differentiate into the invasive, giant cell trophoblast, and exhibit properties similar to the endovascular subtype [16].

The early stages of pregnancy take place under low-oxygen conditions prior to complete development of the functioning placenta. Low oxygen (3%) has been shown to maintain the trophoblast stem cell state and inhibit trophoblast differentiation, and several mediators have been implicated [22–26]. Previous studies in our laboratory have demonstrated that low oxygen (1%–3%) inhibits trophoblast giant cell differentiation [16]. While 3% oxygen would be a hypoxic state to most mammalian cells, trophoblast stem cells begin normal development in vivo in oxygen concentrations of ~2%–3%. In vivo, invasive trophoblast cells initially proliferate and mediate blastocyst attachment under low-oxygen conditions. As pregnancy progresses, invasive trophoblast cells migrate into the uterine tissues and gain access to the uterine arteries. Thus, the differentiation of invasive trophoblast cells takes place in an environment in which they encounter a gradient of oxygen from 2% to 3% in the uterine decidua to ~10% to 12% in the arteries [26–30].

Numerous cellular responses to low oxygen are mediated at the molecular level by the transcriptional activity of hypoxia-inducible factors (HIFs). HIFs are members of the bHLH-PAS family of transcription factors. HIF1, the major regulator of the cellular responses to hypoxia, consists of an oxygen-sensitive subunit, HIF1alpha (HIF1A), and an oxygen-insensitive subunit, HIF1beta (officially known as the arylhydrocarbon receptor nuclear transporter [ARNT]). While the mRNA for both genes is constitutively and ubiquitously expressed and transcribed, the HIF1A protein is rapidly degraded in the presence of oxygen. The half-life of HIF1A under ambient oxygen conditions is less than 5 min [12, 31–36]. The heterodimeric HIF binds DNA sequences that contain hypoxic response elements (HREs). HIF1A-dependent transcriptional activation results in the upregulation of target genes that promote cell survival during low-oxygen conditions [34, 35, 37]. Another HIF family member, HIF2, officially known as endothelial PAS domain protein (EPAS) 1, consists of an alpha subunit (HIF2A, which is highly similar to the HIF1A subunit and is

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TABLE 1. PCR primer sequences used for RT-PCR and genomic PCR analysis.

Gene	Forward sequence	Reverse sequence	Annealing temp. (°C)	Expected size (bp)	Reference
<i>Id2</i>	5'TCTGAGCTTATGTCGAATGATAGC3'	5'CACCAGCATTCAGTAGGCTCGTGTC3'	62	497	[52]
<i>CMV^a</i>	5'AATGGGCGTGGATAGGCGGTTTGA3'		60		
<i>Hif1a^a</i>	5'GCTCATCAGTTGCCACTTCC3'	5'CGCTGRGTGTTTWTGTTCTT3'	58	373	
<i>Actb</i>	5'ATCGTGGCCGCCCTAGGCA3'	5'TGGCTTAGGGTTCAGAGGGG3'	55–65	243	[15]

^a PCR primers were identified by NCBI Blast analysis using MacVector Software.

also regulated by oxygen levels) and ARNT. HIF2A, however, exhibits restricted tissue-specific expression [38–41]. Loss of HIF2A expression in mouse models results in embryonic lethality predominantly due to improper development of the vasculature, but does not affect placental development [39]. HIF1A and ARNT knockout mouse models, however, provide evidence that HIF1 activity is essential for placental development and embryonic survival, and aberrant HIF induction has been associated with pre-eclampsia [42–45].

The goals of this study were to examine the hypothesis that HIF1A is a critical mediator of trophoblast differentiation by determining the effects of gradient oxygen levels, which mimic in vivo exposure, on the differentiation of trophoblast giant cells, and to examine the expression and activity of the critical molecular mediator of the mammalian responses to low oxygen, HIF1A.

MATERIALS AND METHODS

Materials

The Rcho-1 rat trophoblast cell line was a kind gift of Dr. Michael Soares, Kansas University Medical Center (Kansas City, KS). The Cos7 cell line was purchased from American Type Culture Collection. RPMI 1640 and Dulbecco modified Eagle medium cell culture media were purchased from Mediatech, Inc. NCTC 135 cell culture media, sodium pyruvate, heparin, human recombinant fibroblast growth factor (FGF)-4, G418 antibiotic, Hoechst dye, and CellLytic NuCLEAR Extraction Kit were obtained from Sigma. Primers were synthesized by Invitrogen. A plasmid used as a positive control for RT-PCR primer specificity, pCDNA3-HA-wild-type HIF1A, was a kind gift of Dr. Frank Bunn, Harvard University (Cambridge, MA). Primary rabbit polyclonal antibody that recognizes PRL3D1 (PL1) was a generous gift of Dr. Michael Soares [17]. Primary polyclonal anti-HIF1A antibodies (NB100–499) were obtained from Novus Biologicals. A plasmid encoding for a constitutively stable form of HIF1A, which was used as a positive control for Western blot analysis and for the generation of HIF1A-3xSDM clones, was a kind gift of Dr. Christine Warnecke, Universität Erlangen-Nürnberg (Erlangen-Nürnberg, Germany) [46]. Primary monoclonal antibody that recognizes palladin was a generous gift of Dr. Carol Otey, University of North Carolina at Chapel Hill (Chapel Hill, NC) [47]. The monoclonal antibody that recognizes pan-actin was a gift of Dr. James Lessard, Cincinnati Children's Hospital Medical Center (Cincinnati, OH) [48]. Secondary antibodies were purchased from BD Transduction Laboratories (anti-rabbit) or Promega (anti-mouse). Rhodamine-conjugated Phalloidin (R415) and FluorSave mounting media were obtained from Calbiochem and Molecular Probes, respectively. Metafectene transfection reagent was purchased from Biontex [49]. The phosphoglycerate kinase 1 (PGK1)-HRE-luciferase reporter plasmid containing six copies of the HRE consensus sequence from the promoter of the PGK1 gene upstream of a firefly luciferase gene in the pGL3 vector was a kind gift of Dr. Peter Ratcliffe, University of Oxford (Oxford, UK) [50]. The constitutive cytomegalovirus (CMV)-promoter upstream of the *renilla* luciferase gene was a kind gift of Dr. Håkan Axelsson, Lund University (Malmö, Sweden) [51]. The Dual Luciferase Reporter Assay Kit was obtained from Promega.

Methods

All experiments were performed independently, a minimum of three times.

Cell culture. Rcho-1 cells were cultured and differentiated as previously described [16, 17]. Rcho-1 cells were maintained in standard media containing RPMI 1640 with L-glutamine supplemented with 20% v/v fetal bovine serum (FBS), 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES (standard media), and antibiotic-antimycotic, and passaged at subconfluency. Cells were cultured in humidified, ambient air supplemented with carbon

dioxide (21% oxygen/5% carbon dioxide) at 37°C. For differentiation experiments, unless otherwise indicated, cells were cultured as described above for 3 days until approximately 75% confluent. Media were changed to differentiation media (NCTC 135 supplemented with 26 mM sodium bicarbonate, 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, and antibiotic-antimycotic) supplemented with 10% v/v horse serum in place of the FBS and denoted as Day 0 of differentiation. Differentiation was induced for 7 days, and differentiation media were replenished on Days 1, 3, and 5 of the differentiation time course. For low-oxygen experiments, cells were incubated in a self-contained, humidified hypoxia incubator (no. 8375-110 Hypoxic Glove Box; Coy Laboratory Products, Inc.), maintained at the described oxygen concentration by an oxygen regulator and supplemented with 5% carbon dioxide, and balanced by nitrogen at 37°C throughout the entire experiment. This hypoxia incubator/chamber contains a double-locking door and gloved ports to allow for media changes and passage while maintaining the cells at the specified oxygen concentration under examination. Reagents used in low-oxygen experiments were pre-equilibrated in the chamber for 30 min prior to use. Pre-equilibration of reagents for longer times (>2 h) produced identical results (data not shown). Control samples were maintained in a humidified incubator supplemented with 5% carbon dioxide in ambient air (21% oxygen) at 37°C. While oxygen concentrations of 21% would be considered physiologically hyperoxic for trophoblasts, nearly all of the previous studies examining Rcho-1 differentiation have been performed under these ambient conditions.

RNA extraction and RT-PCR. Gene expression in cells differentiated at various oxygen concentrations was assessed using RT-PCR as previously described [16, 21]. Rcho-1 cells were maintained in differentiation media for 7 days, and total RNA was extracted from cell pellets using RNeasy RNA Extraction Kit (Qiagen) per the manufacturer's instructions. To prevent amplification of any contaminating residual DNA, total RNA was treated with DNase I. Reverse transcription was performed using 5 μ g RNA, 1 μ g random hexamer primers, 100 μ M deoxyribonucleotide triphosphates, and 50 units Stratascript Reverse Transcriptase. RT product was used as a template for PCRs that contained either 200 μ M deoxyribonucleotide triphosphates, 1.87 mM MgCl₂, 1 unit Taq polymerase, and 10 $\times$ buffer B, or 12.5 μ l 2 $\times$ GoTaq Green along with 200 nM forward primer, 200 nM reverse primer, 40 nM *Actb* forward primer, and 40 nM *Actb* reverse primer [19]. The *Actb* primers were included in each reaction to act as an internal control. Primer sequences for each target gene and the appropriate annealing temperatures are shown in Table 1 [15, 52]. PCR was performed as follows: a 5-min denaturation step at 94°C, 40 cycles of 1 min at 94°C, 1 min of annealing at the primer-specific temperature, and 1 min extension at 72°C, followed by a final extension for 15 min at 72°C [16, 19]. After separation on 1.1% agarose gels, RT-PCR products were visualized with ethidium bromide and ultraviolet light, and images were captured using an LAS-3000 Imager (Fuji) and Image Reader software (Fuji). RT-PCR analysis of HIF1A expression in Rcho-1 cells was performed as described above.

Western blot analysis. For palladin protein analysis, cells were gently scraped in boiling Laemmli reducing buffer [53]. Equal volumes of whole-cell lysates separated by SDS-PAGE and transferred overnight to polyvinylidene fluoride membrane, as previously described [54]. Blots were blocked with 5% nonfat, dry milk in 1 $\times$ PBS containing 0.05% Tween-20 and then incubated with a monoclonal primary antibody that recognizes palladin (1:1000 dilution), followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody [16]. Proteins were visualized by chemiluminescence and exposed to film. To ensure equal loading, pan-actin protein levels were also assayed [48].

Nuclear (HIF1A) and cytoplasmic (PRL3D1, also known as CSH1) extracts were isolated in the appropriate low-oxygen condition, using the CellLytic NuCLEAR Extraction Kit per the manufacturer's instructions, in the absence of protease or proteasome inhibitors. Protein concentrations were determined by the Bradford method [55]. Samples were boiled for 10 min in Laemmli reducing buffer. SDS-PAGE and Western blotting of nuclear and cytoplasmic extracts were performed as described above. Incubation with the primary antibody (polyclonal anti-HIF1A, 1:1000 dilution; polyclonal anti-PRL3D1, 1:1000) occurred overnight at 4°C, followed by incubation with the appropriate

FIG. 1. Low levels of oxygen inhibit the molecular differentiation of Rcho-1 cells. **A**) Expression of the stem cell marker *Id2* (497 bp) was examined using RT-PCR analysis in Rcho-1 cells differentiated at the indicated levels of oxygen. Primers specific for *Actb* (243 bp) were used as an internal control. M indicates 100 bp DNA MW marker. **B**) Levels of cytoskeletal protein palladin in Rcho-1 cells differentiated in the indicated levels of oxygen. Cell lysates collected in boiling Laemmli buffer were analyzed by Western blot using monoclonal antibodies. Equal loading was confirmed by Ponceau staining. **C**) PRL3D1 protein levels in differentiated Rcho-1 cells were analyzed by Western blot; 100 μ g of cytoplasmic protein, obtained from Rcho-1 cells differentiated in indicated levels of oxygen, were probed with polyclonal antibodies to PRL3D1. A nonspecific, cross-reactive band, insensitive to low oxygen, was used as a loading control.

horseradish peroxidase-conjugated, secondary antibody (1:10000). Proteins were visualized as described above.

Cell morphology analysis. Rcho-1 cells (1×10^5 cells) were plated on 60 mm plates, cultured for 3 days to approximately 75% confluency, and differentiated as described above at the indicated oxygen concentration for 7 days. Differentiated cells were fixed in 3.7% paraformaldehyde and stained with rhodamine-conjugated phalloidin followed by Hoechst dye, as previously outlined [16]. Using FluorSave mounting media, glass cover slips were mounted on the plate surface, and staining was observed by epifluorescence microscopy using a Nikon Eclipse TE2000–5 phase contrast microscope. Color overlay images were created using Metamorph software. Images shown were captured at 40 $\times$ magnification. Experiments were performed a minimum of three independent times, and representative examples are shown.

Generation of Rcho-1 cells stably expressing green fluorescent protein or HIF1A-3xSDM. Rcho-1 cells were plated at 1×10^5 cells per 60-mm plate in Rcho-1 standard media containing 20% FBS, as described above. At 24 h postseeding, cells were transfected with either 6 μ g of pEGFP-N1 plasmid or 6 μ g of HIF1A-3xSDM plasmid using 6 μ l of Metafectene transfection reagent in serum-free media. The sequence for HIF1A in the HIF1A-3xSDM plasmid contains two point mutations that alter key amino acids necessary for the hydroxylations that mediate the degradation of HIF1A protein in the presence of oxygen, and an additional mutation that prevents hydroxylation of a third amino acid, an inhibitory posttranslational modification, rendering the protein stable and thus active in ambient oxygen [46]. The HIF1A-3xSDM plasmid contains a G418 eukaryotic selectable marker, which was used for isolation of stable, expressing clones. After 18 h, media were changed to the standard, serum-containing media. At 48 h posttransfection, cells were placed under selection with standard media containing 0.75 mg/ml G418 antibiotic. Cells were selected in G418 for 7 days, with media changes containing fresh antibiotic every other day. Stable clones expressing HIF1A-3xSDM or green fluorescent protein (GFP) were isolated by limiting dilution with continued antibiotic selection. GFP-expressing clones were confirmed by fluorescence microscopy, and HIF1A-3xSDM-expressing clones were confirmed by genomic PCR analysis, Western blot analysis, and luciferase reporter assays.

Genomic PCR. Genomic DNA from Rcho-1 clones stably expressing GFP or HIF1A-3xSDM was isolated from cell pellets using DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. PCR analysis was performed using constant volumes of 200 nM CMV forward primer, 200 nM *Hif1a* reverse primer, 40 nM *Actb* forward primer, 40 nM *Actb* reverse primer, and 2 $\times$ GoTaq Green (Promega) mix. See Table 1 for *Actb* primer sequences. Genomic PCR was performed as follows: a 5-min denaturation step at 94 $^{\circ}$ C, 40 cycles of 1 min at 94 $^{\circ}$ C, 1 min of annealing at 60 $^{\circ}$ C, and 1 min extension at 72 $^{\circ}$ C, followed by a final extension for 15 min at 72 $^{\circ}$ C [16, 19].

Luciferase reporter assays. To determine HRE reporter activity in differentiated Rcho-1 cells cultured under graded oxygen conditions, Rcho-1 cells were transfected with 1 μ g PGK1-HRE luciferase reporter plasmid and 0.2 μ g CMV promoter constitutive reporter plasmid using 5 μ l Metafectene reagent, as previously described [21, 49, 56]. To control for transfection efficiency in experiments performed in low oxygen, 2 days posttransfection, each transfection was split into two samples of equal cell number. Samples

were carried for an additional 3 days under nondifferentiating conditions, and subsequently differentiated for 7 days in either ambient 21% oxygen or the indicated level of oxygen. On differentiation Day 7, luciferase reporter activity was determined according to the manufacturer's instructions. Results show the fold increase in HRE activity in samples cultured in low oxygen relative to the activity in corresponding controls (set to a value of 1) from the same transfections maintained in ambient oxygen.

To determine HRE reporter activity in differentiated HIF1A-3xSDM- or GFP-expressing clones, cells were transfected with 1 μ g PGK1-HRE luciferase reporter plasmid and 0.2 μ g pRLSV40-promoter constitutive reporter plasmid, as described above. PGK1 is a direct target of HIF1A [46]. All clones were maintained in ambient oxygen and analyzed for luciferase activity 42 h posttransfection. In each sample, HRE promoter activity results were normalized to constitutive CMV promoter activity to control for transfection efficiency. Results show the fold increase in HRE reporter activity in HIF1A-3xSDM-expressing clones compared to GFP-expressing clones (set to a value of 1).

All luciferase reporter results are an average of a minimum of three independent experiments. Error bars represent SD, and significance ($P < 0.05$) was determined using the Student *t*-test. To determine statistical significance, HRE reporter activation under 3%, 5%, or 8% oxygen was compared with the corresponding control from the same transfection maintained in ambient (21%) oxygen only. For clarity, the fold changes in luciferase reporter activation observed under each of the various oxygen concentrations are shown in one figure. Similarly, for HRE reporter activation in the HIF1A-overexpressing clones (H2, H4, and H6), luciferase reporter activation in each clone was compared with a GFP-expressing control (G), and we have represented the fold changes in reporter activation observed in various clones in one figure for clarity.

RESULTS

To appropriately describe the results of our study, the term "low oxygen" (3%) is used to refer to the environment in which trophoblast stem cells normally exist in vivo. The terms "hypoxic" and "normoxic," in reference to the study of trophoblast stem cells and giant cell differentiation from a trophoblastic perspective, are inaccurate descriptors, as the trophoblasts are not likely to encounter or be exposed to a hypoxic or normoxic state, but only to a gradient of oxygen levels.

While the initial phases of embryonic implantation occur in oxygen concentrations of 2%–3% oxygen, trophoblast giant cells become exposed to an increasing gradient of oxygen as they migrate toward the uterine arteries. In this context, we indicate that low oxygen (3%) is a reduced oxygen concentration, relative to what levels may be present ($\sim$ 12%) after the first trimester. In this study, differentiation of Rcho-1

FIG. 2. Low levels of oxygen inhibit the morphological differentiation of Rcho-1 cells. Stress fiber formation in Rcho-1 cells differentiated in indicated levels of oxygen was examined by staining differentiated cells using Rhodamine-conjugated Phalloidin (red). Nuclei were distinguished with Hoechst dye (blue). Stained Rcho-1 cells were analyzed for stress fiber formation using epifluorescence microscopy at 40 $\times$ magnification. Bar = 100 μ m. Stress fibers are indicated by white arrows.

trophoblast cells under graded oxygen concentrations was examined to determine if physiologically relevant levels of graded oxygen would have an effect on the differentiation process (Fig. 1). The oxygen concentrations selected were based on levels reported during human placental development [29]. Differentiation of Rcho-1 cells to the giant cell phenotype is typically induced under ambient oxygen (21% oxygen) conditions, and results in the downregulation of the trophoblast stem cell marker, *Id2*.

For this study, *Id2* expression was examined by RT-PCR in Rcho-1 cells differentiated in gradient oxygen for 7 days (Fig. 1A). Differentiation of Rcho-1 cells in 21% oxygen (ambient oxygen) dramatically downregulates the expression of *Id2*. Likewise, differentiation in 5% and 8% oxygen (as well as 12% oxygen [data not shown]) downregulated *Id2* mRNA levels. In contrast, *Id2* mRNA was detected in Rcho-1 cells cultured under differentiating conditions for 7 days in 3% oxygen. These results indicate that Rcho-1 cells maintained their stem cell-like profile in low oxygen (3% oxygen); however, exposure to increasing levels of gradient oxygen (5% and 8%) prevents *Id2* expression.

In addition to the changes observed in mRNA expression, differentiation of trophoblast giant cells is also characterized by changes in cell morphology. One step in this process is the production of palladin, a phosphoprotein associated with focal adhesions and an essential mediator of stress fiber formation [47]. Palladin protein levels were examined by Western blot analysis in whole-cell lysates of Rcho-1 cells differentiated in 3%–8% oxygen (Fig. 1B); 3% oxygen showed no detectable or background levels of palladin protein. In contrast, Rcho-1 cells differentiated at both 5% and 8% oxygen showed increasing levels of palladin protein. Palladin protein levels were the highest in ambient oxygen.

Another hallmark of differentiated trophoblast giant cells is their production of the PRL3D1 (previously known as PL1 and CSH1), a placental hormone [15]. To examine the effect of graded oxygen on PRL3D1 protein levels, PRL3D1 protein was examined by Western blot analysis (Fig. 1C). Upon differentiation in 21% oxygen, PRL3D1 protein (40 kDa) was detected in Rcho-1 cytoplasmic extracts. In contrast, differen-

tiation in 3%, 5%, or 8% oxygen failed to induce PRL3D1 protein levels. Culturing Rcho-1 cells in 12% oxygen also failed to induce PRL3D1 protein (data not shown).

The effect of graded oxygen on the differentiation of Rcho-1 cells was also assessed by examining the formation of cytoskeletal stress fibers, which are assembled during the morphological differentiation of trophoblast giant cells. Rcho-1 cells differentiated in gradient oxygen concentrations were stained with rhodamine-conjugated phalloidin, which binds to filamentous actin, the predominant component of stress fibers (Fig. 2). Our data demonstrate that 3% and 5% oxygen inhibited the formation of stress fibers in differentiating Rcho-1 cells, while Rcho-1 cells differentiated in 8% oxygen developed low levels of stress fibers. Although the level of stress fiber induction was substantially less than that observed in cells cultured in 21% oxygen, 8% oxygen was permissive for the initial induction of stress fiber assembly. The data show that palladin protein induction occurs at a lower oxygen concentration than the level required for stress fiber formation, suggesting that palladin protein accumulation occurs as an earlier step in the morphological differentiation of trophoblast giant cells, followed by the formation of stress fibers at a later stage of differentiation. Thus, trophoblast giant cell differentiation appears to undergo an established order of events that is regulated by exposure to an oxygen gradient.

To determine the molecular mechanism of low-oxygen inhibition of differentiation, the expression of *Hif1a*, the major mediator of the cellular hypoxic response, was examined. Rcho-1 cells differentiated in 3%, 5%, 8%, and 21% oxygen constitutively expressed *Hif1a* mRNA (Fig. 3A). These results are in agreement with other reports that *Hif1a* mRNA expression is ubiquitous and constitutive. Since the regulation of HIF activity is controlled in large part by modulation of HIF protein stability, we next examined the levels of HIF1a protein in Rcho-1 cells differentiated in oxygen levels between 3% and 21% (Fig. 3B). Western blot analysis of nuclear extracts revealed that the highest levels of HIF1A protein were detected in Rcho-1 cells differentiated in 3% oxygen. Decreasing levels of HIF1A were detected in 5% oxygen, while little to no protein was observed in 8%, 12%, or 21% oxygen (Figure 3B).

FIG. 3. Low oxygen induces HIF1A activity in Rcho-1 cells. **A)** *Hif1a* mRNA expression was analyzed using RT-PCR with primers specific for *Hif1a* (373 bp). Primers specific for *Actb* (243 bp) were used as an internal control. M indicates 100bp DNA MW marker. P₁, HIF1A plasmid. **B)** HIF1A protein in Rcho-1 cells differentiated at the indicated levels of oxygen was analyzed by Western blot using polyclonal antibodies to HIF1A protein. Actin was used as a loading control. **C)** Luciferase reporter assays were used to detect HIF1 activity in Rcho-1 cells. Cells were transfected with the pGL3-6X-PGK1-HRE-luciferase reporter plasmid and differentiated for 7 days at the indicated oxygen levels. Activity in low oxygen is shown relative to reporter activity in 21% ambient oxygen control samples, which was set at 1 (mean $\pm$ SD, n = 3). Significance (* $P \leq 0.05$) was determined using Student *t*-test; error bars represent SD.

and data not shown). The presence of HIF1A protein, however, may not directly relate to HIF transcriptional activity, because HIF activity is tightly regulated, not only by rapid turnover of HIF1A when oxygen is abundant, but also by posttranslational modifications [32–34, 57, 58]. In order to determine whether the HIF1 protein is transcriptionally active in Rcho-1 cells differentiated in 3% low oxygen, Rcho-1 cells were transfected with a luciferase reporter plasmid in which HREs from the murine PGK1 promoter were placed upstream of a luciferase reporter gene [50]. PGK1 has been shown to be a direct target of HIF1 [46, 59]. Following transfection, differentiation was induced for 7 days in the oxygen concentrations indicated. The PGK1-HRE-lux reporter showed little to no basal level activity in Rcho-1 cells differentiated in ambient oxygen (Fig. 3C). Rcho-1 cells differentiated in 3% oxygen, however, showed a 16.6-fold (± 6.2 ; $P = 0.0023$) increase in PGK1-HRE-lux reporter activity compared with Rcho-1 cells differentiated in 21% oxygen. Rcho-1 cells differentiated in 5% oxygen exhibited a 4.1-fold (± 1.0 ; $P = 0.0007$) increase in activity, while those differentiated in 8% oxygen had only a 2.2-fold (± 0.4 ; $P = 0.0072$) increase over normoxic controls. Since previous studies have identified PGK1 as an HIF1-specific target gene, these data suggest that HIF1 is transcriptionally active in Rcho-1 cells cultured in low oxygen (3%), but that this activity is significantly downregulated with increasing oxygen levels, in parallel with the decrease in HIF1A protein.

Since a decrease in the levels of HIF1A protein correlated with the increase in the level of differentiation, we hypothesized that HIF1A may be a potential mediator of inhibition of

trophoblast giant cell differentiation in low oxygen. To test this, we transfected undifferentiated Rcho-1 cells with a plasmid encoding either a GFP under the control of a CMV promoter (CMV-eGFP) or an oxygen-insensitive—and therefore constitutively active—form of HIF1A under the same promoter (CMV-HIF1A-3xSDM) [46]. Site-directed mutations in the HIF1A sequence of CMV-HIF1A-3xSDM result in a protein that contains three amino acid substitutions that eliminate key regulatory hydroxylation sites, inhibiting the protein's turnover in ambient oxygen and rendering it constitutively active in ambient oxygen. Stable expression of the control CMV-GFP plasmid in GFP-expressing clones was confirmed by fluorescence microscopy (Fig. 4). Genomic integration of the CMV-HIF1A-3xSDM plasmid sequence in HIF1A-3xSDM-expressing clones was confirmed by PCR analysis of genomic DNA using a 5' primer specific for a sequence unique to the plasmid in conjunction with a 3' primer specific for a unique *Hif1a* sequence. Integration and expression of the plasmid was confirmed in several clones (Fig. 5A and data not shown).

Expression of the transfected, constitutively active form of HIF1A was confirmed by Western blot analysis of nuclear extracts of HIF1A-3xSDM-expressing clones using a polyclonal antibody that recognizes both the endogenous, wild type and the integrated, constitutively active form of the protein (Fig. 5B). Three HIF1A-3xSDM clones, H2, H4, and H6, which expressed low, medium, and high levels of the constitutively active protein, were selected for further studies. While clone H2 showed similar levels of HIF1A protein

FIG. 4. Confirmation of GFP-expressing clones. Rcho-1 cells stably expressing either pEGFP-N1 (G) or HIF1A-3xSDM (H2, H4, H6) were analyzed for GFP expression using epifluorescence microscopy (upper panels). Corresponding bright-field photos (lower panels) are shown as controls. Original magnification $\times 40$; bar = 100 μ m.

FIG. 5. Confirmation of HIF1A-3xSDM-expressing clones. **A**) Genomic PCR analysis of Rcho-1 cells stably expressing either pEGFP-N1 (G) or HIF1A-3xSDM (H2, H4, and H6). M, 1 Kb DNA molecular weight marker; P1, HIF1A-3xSDM plasmid-only control; R, undifferentiated Rcho-1 stem cells. PCR was performed using a CMV promoter 5' forward primer and a *Hif1a* 3' reverse primer. *Beta actin* (243 bp) was used as a control. **B**) Western blot analysis of HIF1A protein levels in clones stably expressing pEGFP-N1 (G) or HIF1A-3xSDM (H2, H4, and H6). Nuclear extracts were probed using polyclonal antibodies to HIF1A. Actin protein levels were also analyzed to confirm equal loading. Rcho-1 (labeled Rcho) cells treated with cobalt chloride (CoCl₂) and Cos7 cells expressing the HIF1A-3xSDM plasmid (Cos7) were used as positive controls for HIF1A detection. **C**) HRE luciferase reporter activity in Rcho-1 cells stably expressing either pEGFP-N1 (G) or HIF1A-3xSDM (H2, H4, and H6). Clones were transfected with the pGL3-6X-PGK1-HRE-luciferase reporter plasmid and a constitutively activated control reporter (CMV promoter) to control for transfection efficiency. Cells were cultured in ambient oxygen. Luciferase activity in HIF1A-3xSDM clones is shown relative to activity in GFP clones, which was set at 1. Data presented are means $\pm$ SD; n = 3.

compared with GFP-expressing clones in ambient oxygen, HIF1A levels in ambient oxygen were markedly increased in clones H2, H4, and H6. In addition, HIF1A transcriptional activity in HIF1A-3xSDM clones was analyzed by PGK1-HRE luciferase reporter assays performed in ambient oxygen (Fig. 5C). As occurs in wild-type Rcho-1 cells, the PGK1-HRE-lux reporter showed little or no basal level activity in ambient oxygen in the GFP-expressing clone. Stable expression of the constitutively active HIF1A (in clones H2, H4, and H6), however, upregulated the activity of the PGK1-HRE luciferase reporter by 2- to 3-fold over activity in GFP-expressing clones. This shows that, although clone H2 showed similar levels of HIF1A protein when compared to the GFP clone control, the H2 clone is expressing a stable (hydroxylation-resistant), active form of the protein, while the low level of endogenous (and rapidly degraded) HIF1A protein observed in the GFP clones is not active. The rapid degradation of endogenous HIF1A protein in the GFP-expressing clone likely limits its ability to transcriptionally activate, whereas, in the constitutively active HIF1A clones, the protein is free to be active. This analysis demonstrates that the oxygen-insensitive HIF1A-3xSDM protein is expressed and active in the HIF1A-3xSDM-expressing Rcho-1 clones, H2, H4, and H6.

To determine whether constitutively active HIF1A expression altered the differentiation of Rcho-1 cells to the trophoblast giant cell phenotype, differentiation was induced in HIF1A-3xSDM-expressing clones H2, H4, and H6 in ambient oxygen (21% oxygen) for 7 days. Differentiation was also simultaneously induced in GFP-expressing Rcho-1 clones as controls. As expected, GFP-expressing clones exhibited the hallmark features of differentiated Rcho-1 cells (Figs. 6 and 7). RT-PCR analysis demonstrated that GFP-expressing cells downregulated the expression of *Id2* on Day 7 of differentiation when compared with undifferentiated cells (Fig. 6A). HIF1A-3xSDM clones H2, H4, and H6 cultured under differentiating conditions, however, continued to express

detectable levels of *Id2* mRNA, a marker of trophoblast stem cells, suggesting an inhibition in the differentiation process in the presence of active HIF1A. Western blot analysis of the control GFP-expressing clones also showed the expected increase in palladin protein levels upon induction of differentiation (Fig. 6B). In contrast, HIF1A-3xSDM-expressing cells exhibited variable but consistently lower levels of palladin protein when compared with GFP-expressing controls. As previously mentioned, the functional differentiation of trophoblast giant cells is also marked by the production of PRL3D1 protein. Only differentiated GFP-expressing clones produced detectable levels of PRL3D1 protein (Fig. 6C). HIF1A-3xSDM clones H2, H4, and H6 cultured under differentiating conditions in ambient oxygen did not produce PRL3D1 protein. Moreover, GFP-expressing cells differentiated in ambient oxygen exhibited normal differentiation-induced stress fiber formation, as indicated by phalloidin staining (Fig. 7G), while HIF1A-3xSDM-expressing clones did not adopt the morphological phenotype of differentiated trophoblast giant cells (Fig. 7, H2, H4, H6).

The HIF1A-3xSDM-expressing clonal cell lines, H2, H4, and H6, are the first models of placental differentiation to examine the effect of constitutive HIF1A expression on trophoblast giant cell differentiation. This study suggests that the inhibitory effect of gradient oxygen levels on the differentiation of trophoblast giant cells is directly mediated by HIF1A.

DISCUSSION

In this study, in order to more closely mimic the gradient of oxygen concentrations invasive trophoblast cells encounter as they migrate and invade in vivo, we investigated the effects of gradient oxygen levels on the differentiation process of Rcho-1 trophoblast cells. Several aspects of trophoblast differentiation, including the expression of trophoblast stem cell marker *Id2*,

FIG. 6. HIF1A-3xSDM expression inhibits the molecular differentiation of Rcho-1 cells. Top: RT-PCR analysis of stem cell marker *Id2* (497 bp) in undifferentiated (U) and differentiated (D) Rcho-1 clones expressing either pEGFP-N1 (G) or HIF1A-3xSDM (H2, H4, H6) cultured in ambient oxygen for 7 days. Primers specific for *Actb* (243 bp) were used as internal controls. Untransfected, undifferentiated Rcho-1 stem cells (R) were used as a positive control for *Id2* expression. M, DNA molecular weight marker. Bottom left: levels of cytoskeletal protein palladin in Rcho-1 clones stably expressing either pEGFP-N1 (G) or HIF1A-3xSDM (H2, H4, H6). Clones were cultured under differentiating conditions in ambient oxygen for 7 days. Equal volumes of cell lysates collected in boiling Laemmli buffer were analyzed by Western blot using monoclonal antibodies. Equal loading was confirmed by Ponceau staining. Bottom right: Western blot analysis of protein levels using polyclonal antibodies that recognize PRL3D1. Rcho-1 clones were cultured under differentiating conditions in ambient oxygen for 7 days, and PRL3D1 protein levels in cytoplasmic fractions of both GFP-expressing (G) and HIF1A-3xSDM-expressing (H2, H4, H6) clones were analyzed. A nonspecific, cross-reactive band, insensitive to low oxygen, was used as a loading control.

the induction of palladin protein, the formation of stress fibers, and the induction of PRL3D1 protein, were examined. Our data demonstrate that conditions of 3% oxygen had the same effect on trophoblast giant cell differentiation as in our previous study in 1% oxygen: sustained expression of *Id2*, inhibition of PRL3D1 and palladin protein induction, and prevention of stress fiber formation. Although giant cell indicators were present, we are not able to discern whether low oxygen may possibly alter the placental lineage of the Rcho-1 cells due to the lack of other rat placental cell lines that would serve as positive controls. Nevertheless, low oxygen (3%) prevented all of the key features of trophoblast giant cell differentiation at the molecular, morphological, and functional level.

Increasing oxygen concentrations to 5%, 8%, and 12% oxygen, however, allowed for specific aspects of differentiation to occur. These intermediate levels of oxygen did allow for low

levels of molecular and morphological differentiation, but they did not induce PRL3D1 protein levels, a marker of the differentiated state [15]. This has led us to classify 5% and 8% as “intermediate” oxygen levels. Intermediate oxygen levels are permissive for low levels of molecular and morphological differentiation, but still suppress the extent of differentiation observed in ambient oxygen conditions or in oxygen concentrations equivalent to arterial oxygen levels. The higher level of oxygen signaling required to induce differentiation of Rcho-1 cells may indicate that this particular trophoblast giant cell subtype may need to reach the higher oxygen concentrations in order to complete the differentiation process.

The gradient oxygen concentrations used in this study represent physiologically relevant concentrations encountered during trophoblast development. Thus, this model demonstrates that the differentiation of Rcho-1 cells is mediated in a

FIG. 7. HIF1A-3xSDM expression inhibits the morphological differentiation of Rcho-1 cells. Rcho-1 clones expressing either pEGFP-N1 (G) or HIF1A-3xSDM (H2, H4, and H6) were cultured under differentiating conditions in ambient oxygen for 7 days. Stress fiber formation was examined using Rhodamine-conjugated Phalloidin stain (red). Nuclei were distinguished by Hoechst dye (blue). Stained cells were analyzed for stress fiber formation using epifluorescence microscopy at 40 $\times$ magnification. Bar = 100 μ m. Stress fibers are indicated by white arrows.

concentration-dependent manner by graded oxygen: increasing oxygen allows for increasing differentiation. The transition from the proliferative, stem cell-like population observed in low oxygen (3%) to the levels of differentiation seen in intermediate oxygen (5%–8%) corresponds with the changes detected in invasive trophoblast cells in vivo. Trophoblast cells are undifferentiated in the 3% low-oxygen environment of early pregnancy, and differentiate according to cues encountered throughout the stages of placental development as oxygen exposure increases. This study demonstrates that one particular oxygen concentration does not control differentiation, and that increasing levels of oxygen allow specific steps of differentiation to occur. This progression mimics the process that trophoblast giant cells undergo as they invade and differentiate into mature and fully functional giant cells in response to increasing oxygen levels.

Thus, future analysis of the mediators of placental differentiation should be examined from this new paradigm in which specific levels of oxygen may modulate the level and activity of regulatory proteins. Mediators of differentiation previously identified under ambient oxygen conditions may not play the same role in the lower oxygen environment of early pregnancy. The terminology used to describe studies of trophoblast cells in low oxygen may be in need of refinement, as trophoblast stem cells develop in a 2%–3% low-oxygen environment. While this may be a “hypoxic” environment for most mammalian cells, this is not true of trophoblasts, as 3% oxygen is initially their normal environment. In fact, for trophoblast stem cells, 3% oxygen would be considered “normoxic.” Thus, terminology such as low oxygen level (2%–3% or LOL), intermediate oxygen level (5%–8% or IOL), and arterial oxygen level (12%–14% or AOL) would be more appropriate.

The inhibitory effect of 3% low oxygen on the differentiation process closely paralleled the stabilization and activity of HIF1A. Low oxygen (3%) inhibits trophoblast giant cell differentiation, and this inhibition corresponds with the induction pattern for HIF1A protein. Intermediate oxygen levels (5%–8%), however, allow distinct aspects of differentiation to occur as HIF1A protein levels and transcriptional activity are decreasing. In addition, the HIF1A-3xSDM-expressing cell lines do not show complete differentiation in ambient 21% oxygen, demonstrating that HIF1A mediates the inhibitory effects of low oxygen on the differentiation process. It was observed, however, that later passages of the H4 clone (but not the H2 or H6 clones) had a reduction in the amount of HIF1A protein, suggesting that the integrated gene in this one clone may be silenced over time. Overall, the Rcho-1 HIF1A-3xSDM clonal trophoblast cell lines, which exhibit stem cell-like properties, even under differentiating conditions, provide unique opportunities to investigate the role of HIF1A during the early phases of invasive trophoblast differentiation.

This is the first reported study to examine the effects of constitutive HIF1A activity on the trophoblast differentiation process, and our results suggest that failure to regulate HIF1A could contribute to inhibited differentiation of invasive trophoblast cells. This approach will allow us to look into a multiplicity of factors that could contribute to inappropriate or defective placental development. The distinct roles for HIF1 and other HIF family members in this process are currently under investigation. While other reports have demonstrated that the loss of HIF1A plays a detrimental role in placental development, this new analysis allowed us to demonstrate that inappropriate stabilization of HIF1A can result in prolonged HIF1A activity that could adversely effect trophoblast differentiation, and may ultimately lead to aberrant placental

development with severe pathological outcomes [44]. This is supported by a recent report on *Egln1* knockout mice by Takeda et al. [60]. Prolyl hydroxylase domain proteins (PHDs) are responsible for the rapid degradation of HIFs under ambient oxygen conditions. The absence of PHD's allows for stabilization and activation of HIFs under ambient oxygen levels in the knockout mice. *Egln1*^{−/−} (also known as *Phd2*^{−/−}) mice show defects in placenta formation with significantly reduced numbers and irregular distribution of trophoblast giant cells.

Understanding the functions of oxygen gradients and HIF proteins during the trophoblast differentiation process may lead to new insights into the causes of pre-eclampsia and other pathologies that lead to miscarriage and intrauterine growth retardation. In addition, these studies may lead to the development of diagnostic tools to identify those at risk for these conditions, and potential therapeutic treatments for placental dysregulation.

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