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Hypoxia Inhibits Differentiation of Lineage-Specific Rcho-1 Trophoblast Giant Cells

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ABSTRACT

Defects in placental development lead to pregnancies at risk for miscarriage and intrauterine growth retardation and are associated with preeclampsia, a leading cause of maternal death and premature birth. In preeclampsia, impaired placental formation has been associated with alterations in a specific trophoblast lineage, the invasive trophoblast cells. In this study, an RT-PCR Trophoblast Gene Expression Profile previously developed by our laboratory was utilized to examine the lineage-specific gene expression of the rat Rcho-1 trophoblast cell line. Our results demonstrated that Rcho-1 cells represent an isolated, trophoblast population committed to the giant cell lineage. RT-PCR analysis revealed that undifferentiated Rcho-1 cells expressed trophoblast stem cell marker, Id2, and trophoblast giant cell markers. On differentiation, Rcho-1 cells downregulated Id2 and upregulated Csh1, a marker of the trophoblast giant cell lineage. Neither undifferentiated nor differentiated Rcho-1 cells expressed spongiotrophoblast marker Tpbpa or labyrinthine markers Esx1 and Tec. Differentiating Rcho-1 cells in hypoxia did not alter the expression of lineage-specific markers; however, hypoxia did inhibit the downregulation of the trophoblast stem cell marker Id2. Differentiation in hypoxia also blocked the induction of CSH1 protein. In addition, hypoxia inhibited stress fiber formation and abolished the induction of palladin, a protein associated with stress fiber formation and focal adhesions. Thus, Rcho-1 cells can be maintained as a proliferative, lineage-specific cell line that is committed to the trophoblast giant cell lineage on differentiation in both normoxic and hypoxic conditions; however, hypoxia does inhibit aspects of trophoblast giant cell differentiation at the molecular, morphological, and functional levels.


INTRODUCTION

With the advent of molecular biology and the study of genetics, the ability to identify the causes for miscarriage and other life-threatening complications of pregnancy is increasing. One of the essential contributors to embryonic survival and fetal growth is proper placental development. Errors in placental formation can be catastrophic because the placenta fulfills a variety of functions during pregnancy. Placental cells are responsible for attaching the embryo to the uterine lining, protecting the baby from maternal immunological attack, secreting hormones to maintain the pregnancy, and exchanging nutrients and wastes between mother and baby [1, 2]. Defects in placental development lead to pregnancies at risk for miscarriage and intrauterine growth retardation (IUGR) and are associated with preeclampsia, a leading cause of maternal death. Preeclampsia is characterized by the development of maternal hypertension and can result in IUGR as well as the necessity to induce premature birth. Preeclampsia is estimated to occur in approximately 5% of human pregnancies [3–6]. Investigations into the molecular mediators of placental formation are yielding important information regarding the possible etiologies of many of the complications of pregnancy; however, the functional roles of many essential placental proteins have not been elucidated. Delineating the functions of these proteins often requires genetic and molecular manipulations that are prohibited by the practical and ethical considerations of human placental research. Thus, animal and cell line models are being utilized to answer pertinent questions regarding the formation and function of the placenta [7–11].

Hypoxia

Humans and rodents have analogous placental trophoblast cell types that perform similar functions and express homologous molecular mediators. In the rodent placenta, three major lineages of differentiated trophoblasts arise from trophoblast stem cells: labyrinthine cells, spongiotrophoblasts, and giant cells (Fig. 1) [12, 13]. Trophoblast subtypes can be distinguished from one another by the expression of lineage-specific genetic markers (Table 1). For example, in situ hybridization studies have shown that expression of the transcription factor inhibitor of DNA-binding 2 (Id2) is limited to undifferentiated, proliferative trophoblast cells. ID2 is a helix-loop-helix (HLH) protein that binds other HLH transcription factors but lacks a basic DNA binding domain, inhibiting HLH-mediated transcriptional activity [14, 15]. The loss of Id expression is associated with differentiation in several cell types [16–19]. Labyrinthine cells, which mediate the exchange of nutrients and wastes between mother and baby, have a distinct genetic profile that includes expression of the transcription factors Esx1, Tcfeb, Dlx3, and Gcm1 and the kinase Tec [20–23]. While the functional role of the spongiotrophoblasts remains elusive, they can be identified by their expression of Tbpba (previously identified as Tpbp or 4311) [24, 25]. Expression of Snail (formerly classified as SNA), Hand1, and chorionic somatomammotropin hormone 1 (Csh1; also known as Placental Lactogen1) in the placenta is limited to trophoblast giant cells [26–29]. Messenger RNA for the transcriptional repressor Snail is expressed during the intermediate stage of giant cell differentiation [26]. The HLH transcription factor HAND1 plays an essential role in directing the differentiation of trophoblast stem cells to the giant cell lineage, and loss of Hand1 expression results in embryonic lethality [27, 28, 30]. The secreted hormone CSH1 is a member of the prolactin family of hormones expressed by trophoblast giant cells in order to maintain the pregnancy [31]. Trophoblast
MMP-9 differentiated state by culturing under high serum conditions [26, 30, 31]. Rcho-1 cells are maintained in a proliferative, undifferentiated state, which can be induced to differentiate into functional, trophoblast giant cells. Originally derived from a rat transplantable choriocarcinoma, the Rcho-1 cell line is well characterized and determines its utility as a model to study oxygen’s role in giant cell differentiation.

An invasive phenotype is the hallmark of the trophoblast giant cell. Invasive trophoblasts in both humans and rodents express conserved genes such as STRA13/Stra13, a transcription factor that mediates the response to retinoic acid, a known inducer of trophoblast differentiation, and matrix metalloproteinase 9 (MMP-9/Mmp9), a mediator of the invasive process [13, 28, 32–34]. The extracellular matrix protein receptors alpha-5 integrin and alpha-1 integrin are also expressed in invasive trophoblasts in both humans and rodents [35–37]. Several inducers of trophoblast differentiation have been identified, including changes in extracellular matrix components, hormones from both embryonic and maternal sources, as well as growth factors or the loss of growth factor exposure [10, 38–43]. Failure of the invasive trophoblasts to fully differentiate is associated with preeclampsia [2–6, 44–46].

Low oxygen levels have been demonstrated to act as an inhibitor to trophoblast differentiation [37, 47–56]. For example, studies using human placental explants show that the invasive capabilities of trophoblast cells are inhibited when cultured in a hypoxic environment of 3% oxygen [37]. Hypoxic culturing conditions correlate with the environment of early pregnancy when the placenta is forming but not fully functional. During this time, trophoblast exposure to the maternal circulation is restricted, and the supply of oxygen for trophoblast cells is limited to diffusion from the maternal uterine tissues. Thus, implantation is estimated to occur in an oxygen concentration of approximately 2% [57–59]. While extended hypoxia of this degree will induce apoptosis in most other cell types, trophoblast cells remain proliferative and fulfill their first roles in implantation under low oxygen conditions. As the pregnancy progresses, trophoblast giant cells differentiate, invade the maternal decidua, and migrate toward the uterine spiral arteries [49, 60]. The molecular mechanisms by which oxygen mediates trophoblast differentiation have not been fully elucidated.

This study investigated a rat trophoblast cell line, Rcho-1, to determine its utility as a model to study oxygen’s role in giant cell differentiation. Originally derived from a rat transplantable choriocarcinoma, the Rcho-1 cell line is well characterized and can be induced to differentiate into functional, trophoblast giant cells. Rcho-1 cells are maintained in a proliferative, undifferentiated state by culturing under high serum conditions [26, 30, 31, 43, 61–67]. They express Mmp9, and their invasive capability is demonstrated by their degradation of the in vitro basement membrane Matrigel [64]. Rcho-1 differentiation into giant cells is induced by a change in serum culturing conditions and mimics the in vivo differentiation process of rat trophoblast giant cells [43].

In this study, we evaluated Rcho-1 expression of trophoblast lineage-specific genes to determine if the cell line represents an exclusive trophoblast giant cell population. In addition, we determined if hypoxia inhibits Rcho-1 giant cell differentiation. Using an RT-PCR-based Gene Expression Profile recently developed by our laboratory, Rcho-1 expression of lineage-specific markers was examined, and the effect of hypoxia on differentiation was determined by analyzing changes in gene expression, protein levels, and morphology. Our results demonstrated that Rcho-1 cells express genes exclusively present in the trophoblast giant cell lineage and that hypoxia inhibits aspects of trophoblast giant cell differentiation at the morphological, molecular, and functional levels.

### 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, Kansas. The mouse trophoblast stem cell line TS	extsubscript{3.5} was a kind gift of Dr. Janet Rossant, Mt. Sinai Hospital, Toronto, Ontario, Canada. Primary embryonic fibroblasts (pMEFs) were purchased from Stem Cell Technology, Inc. RPMI 1640 cell culture media was purchased from Mediatech, Inc. NCTC 135 cell culture media, sodium pyruvate, human recombinant Fibroblast Growth Factor-4 (FGF-4), heparin, and Hoescht dye were obtained from Sigma. Hepes buffer, antibiotic-antimycotic, and 0.25% trypsin/EDTA were obtained from Gibco. Supersignal West Pico Chemiluminescent Substrate and 2-mercaptoethanol were purchased from Pierce. RNase-free Dnase I and protease inhibitor cocktail tablets were obtained from Roche. Reverse Transcriptase was purchased from Stratagene. Other reagents for RT-PCR, including random hexamer primers, nucleotides, and Taq polymerase, were obtained from Promega. Primers were synthesized by Invitrogen. Primary polyclonal antibody that recognizes CSH1 was a gift of Dr. Michael Soares. Primary monoclonal antibody that recognizes palladin was a gift of Dr. Carol Otey, University of North Carolina at Chapel Hill. The monoclonal antibody that recognizes pan-actin was a gift of Dr. James Lessard, Cincinnati Children’s Hospital Medical Center. Secondary antibodies were purchased from BD Transduction Laboratories. Kodak X-omat AR film was used for CSH1 Western blot analysis, while palladin expression was analyzed using a Fuji LAS-3000 Imager. Rhodamine-conjugated phalloidin (R415) was obtained from Molecular Probes.

**Methods**

**Cell culture.** Rcho-1 cells were passaged at subconfluency in RPMI 1640 with 1-glutamine supplemented with 20% v/v fetal bovine serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES, and antibiotic-antimycotic as previously described [43]. For differentiation experiments, unless otherwise indicated, cells were cultured as described previously for 3 days until approximately 75% confluent. Then, on Day 0 of differentiation, media were changed to differentiation media: NCTC 135 containing 10% v/v horse serum, 26 mM sodium bicarbonate, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, and antibiotic-antimycotic [43]. After the media change, differentiation was induced for 7 days, unless otherwise indicated. Media were replenished on Days 1, 3, and 5 of the 7-day differentiation time course (Fig. 2).
For hypoxic experiments, cells were incubated in a humidified, hypoxic chamber (Coy Laboratory Products, Inc.) maintained at 1% oxygen/5% carbon dioxide/94% nitrogen at 37°C. In order to decrease cellular exposure to oxygen, reagents used in hypoxic experiments were pre-equilibrated in the chamber for a minimum of 30 min prior to use. Normal culturing conditions were in ambient air supplemented with carbon dioxide (21% oxygen/5% carbon dioxide) at 37°C, and these conditions were used for control samples identified as normoxic. Experiments were performed a minimum of three independent times.

The mouse trophoblast stem cell line TS3.5, which can be induced to differentiate simultaneously into all three trophoblast lineages, was cultured and maintained under proliferative conditions to maintain cells in the undifferentiated state or differentiated as described previously [35]: a 4-min denaturation step at 94°C, 5 cycles of 1 min at 94°C, 1 min of annealing at 65 or 55°C, and a 1 min of extension at 72°C, followed by a final extension for 15 min at 72°C [64]. The PCR scheme for alpha-1 integrin and alpha-5 integrin was performed as previously described [35]: a 4-min denaturation step at 94°C, 5 cycles of 1 min at 94°C, 1 min of annealing at 65 or 55°C, and a 1 min of extension at 72°C and a final extension of 15 min at 72°C. PCR products, with the exception of alpha-5 integrin PCR results, were separated on 1.1% agarose gels and visualized with ethidium bromide and UV light. RT-PCR products for alpha-5 integrin were resolved on a 2% low-melt agarose gels and visualized with ethidium bromide and UV light. For hypoxic experiments, cells were incubated in a humidified, hypoxic chamber (Coy Laboratory Products, Inc.) maintained at 1% oxygen/5% carbon dioxide/94% nitrogen at 37°C. To induce differentiation, FGF-4 and heparin were removed from the media, and cells were cultured for an additional 4 days prior to analysis.

RNA extraction and RT-PCR. To examine Rcho-1 gene expression, cells were cultured under proliferative conditions to maintain cells in the undifferentiated state or differentiated as described previously. Total RNA was extracted from cell pellets using RNeasy RNA Extraction Kit as per manufacturer's instructions. Briefly, cells were washed with 1× PBS and removed from plates using a 0.25% trypsin/EDTA solution. Gentle scraping with a rubber spatula was performed for complete removal of differentiated cells. Cell pellets were used immediately or frozen at −70°C. Extracted RNA was treated with 10 units of RNase-free DNase I for 30 min at 37°C. RT was performed using 5 µg RNA, 1 µg random hexamer primers, 100 µM dNTPs, and 50 units Stratascript Reverse Transcriptase as per manufacturer's instructions. PCR was carried out using RT product as template with 200 µM dNTPs, 1.87 mM MgCl2, 1 unit Taq polymerase, 10× Buffer B, 200 nM forward primer, 200 nM reverse primer, 40 nM beta-actin forward primer, and 40 nM beta-actin reverse primer. Primer sequences for each target gene and the appropriate annealing temperatures are listed in Tables 2 and 3. 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 of extension at 72°C, followed by a final extension for 15 min at 72°C [64]. The PCR scheme for alpha-1 integrin and alpha-5 integrin was performed as previously described [35]: a 4-min denaturation step at 94°C, 5 cycles of 1 min at 94°C, 1 min of annealing at 65 or 55°C, and a 1 min of extension at 72°C, followed by a final extension for 15 min at 72°C. PCR products, with the exception of alpha-5 integrin PCR results, were separated on 1.1% agarose gels and visualized with ethidium bromide and UV light. RT-PCR products for alpha-5 integrin were resolved on a 2% low-melt-point agarose gel. Images were captured using a Fuji LAS-3000 Imager and Image Reader software. Messenger RNA from TS3.5 cells was used as positive controls for spongiotrophoblast and labyrinthine specific gene expression [68].

Western blot analysis. For CSFI-1 protein analysis, cells were rinsed in 1× PBS and gently scraped with a rubber spatula in whole cell lysis buffer D (1 M Tris [pH 7.5], 1% NP40, 10% glycerol, 0.5 M EDTA [pH 8.0], and protease inhibitor cocktail tablet) [69]. Soluble protein concentrations were determined by the Bradford method, and protein samples were boiled for 5 min in Laemmli Reducing Buffer [70, 71]. Equal quantities of protein were separated by SDS-PAGE and transferred to PVDF membrane as previously described [69]. Blots

**TABLE 2.** Trophoblast lineage-specific gene expression RT-PCR profile primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Tm (°C)</th>
<th>Expected size (bp)</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Id21</td>
<td>5’tgctgcctttctccgctctctca3’</td>
<td>62</td>
<td>497</td>
<td>[19]</td>
</tr>
<tr>
<td>Snai11</td>
<td>5’gcgttcctctccgctctctca3’</td>
<td>64</td>
<td>793</td>
<td>[26]</td>
</tr>
<tr>
<td>Hand11</td>
<td>5’ggccgctggcctctctctca3’</td>
<td>62</td>
<td>496</td>
<td>[19]</td>
</tr>
<tr>
<td>Csb1</td>
<td>5’tggctctctctctctctctca3’</td>
<td>64</td>
<td>793</td>
<td>[26]</td>
</tr>
<tr>
<td>Tpbpa</td>
<td>5’tggctctctctctctctctca3’</td>
<td>62</td>
<td>793</td>
<td>[26]</td>
</tr>
<tr>
<td>Eft1</td>
<td>5’tggctctctctctctctctca3’</td>
<td>62</td>
<td>793</td>
<td>[26]</td>
</tr>
<tr>
<td>β-Actin2,3</td>
<td>5’tggctctctctctctctctca3’</td>
<td>62</td>
<td>793</td>
<td>[26]</td>
</tr>
</tbody>
</table>

* Amplification temperature.
1 Conserved rodent and human genes.
2,3 The PCR primers for Tm were identified by NCBI BLAST analysis using MacVector Software.

For hypoxic experiments, cells were incubated in a humidified, hypoxic chamber (Coy Laboratory Products, Inc.) maintained at 1% oxygen/5% carbon dioxide/94% nitrogen at 37°C. In order to decrease cellular exposure to oxygen, reagents used in hypoxic experiments were pre-equilibrated in the chamber for a minimum of 30 min prior to use. Normal culturing conditions were in ambient air supplemented with carbon dioxide (21% oxygen/5% carbon dioxide) at 37°C, and these conditions were used for control samples identified as normoxic. Experiments were performed a minimum of three independent times.

The mouse trophoblast stem cell line TS3.5, which can be induced to differentiate simultaneously into all three trophoblast lineages, was cultured and differentiated as previously described [35]: a 4-min denaturation step at 94°C, 5 cycles of 1 min at 94°C, 1 min of annealing at 65 or 55°C, and a 1 min of extension at 72°C, followed by a final extension for 15 min at 72°C. PCR products, with the exception of alpha-5 integrin PCR results, were separated on 1.1% agarose gels and visualized with ethidium bromide and UV light. RT-PCR products for alpha-5 integrin were resolved on a 2% low-melt-point agarose gel. Images were captured using a Fuji LAS-3000 Imager and Image Reader software. Messenger RNA from TS3.5 cells was used as positive controls for spongiotrophoblast and labyrinthine specific gene expression [68].

Western blot analysis. For CSFI-1 protein analysis, cells were rinsed in 1× PBS and gently scraped with a rubber spatula in whole cell lysis buffer D (1 M Tris [pH 7.5], 1% NP40, 10% glycerol, 0.5 M EDTA [pH 8.0], and protease inhibitor cocktail tablet) [69]. Soluble protein concentrations were determined by the Bradford method, and protein samples were boiled for 5 min in Laemmli Reducing Buffer [70, 71]. Equal quantities of protein were separated by SDS-PAGE and transferred to PVDF membrane as previously described [69]. Blots

**TABLE 3.** RT-PCR primer sequences for conserved invasive trophoblast genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Tm (°C)</th>
<th>Expected size (bp)</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmp9</td>
<td>5’ttgctggcctctctctctctca3’</td>
<td>64</td>
<td>443</td>
<td>[64]</td>
</tr>
<tr>
<td>Stra13</td>
<td>5’tggctgtctctctctctctctca3’</td>
<td>62</td>
<td>596</td>
<td>[19]</td>
</tr>
<tr>
<td>Alpha-5 integrin</td>
<td>5’agctccgctggcctctctctca3’</td>
<td>65/50</td>
<td>275</td>
<td>[35]</td>
</tr>
<tr>
<td>Alpha-1 integrin</td>
<td>5’tggctctctctctctctctca3’</td>
<td>65/50</td>
<td>275</td>
<td>[35]</td>
</tr>
</tbody>
</table>

* H = a or t; Y = c or t.
1 Amplification temperature.
2 The PCR primers for Stra13 were identified by NCBI BLAST analysis using MacVector Software.
3 Denotes PCR scheme with two annealing temperatures (see Materials and Methods).
were blocked with 5% nonfat dry milk in 1× PBS containing 0.05% Tween-20 and incubated with polyclonal rabbit primary antibodies that recognize CSH1 [43]. After incubation with a horseradish peroxidase-conjugated anti-rabbit secondary antibody, proteins were visualized by chemiluminescence and exposed to film. For palladin protein analysis, cells were removed from plates by gentle scraping in boiling Laemmli Reducing Buffer. Equal volumes of lysate were separated by SDS-PAGE and transferred as described previously. Blots were blocked and incubated with a monoclonal primary antibody that recognizes palladin [66]. After incubation with a horseradish peroxidase-conjugated anti-mouse secondary antibody, proteins were visualized by chemiluminescence, and images were captured with a Fuji LAS 3000 Imager using Image Reader software. Western blots were also examined using a monoclonal pan-actin antibody to ensure equal loading [72].

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 previously described in either hypoxia or normoxia. On Day 7 of differentiation, cells were fixed in 3.7% paraformaldehyde in 1× PBS at room temperature for 1 h. After extensive washing, cells were permeabilized in buffer containing 3% goat serum, 250 mM KCL, 20 mM HEPES (pH 7.8), 0.1% glycine, and 0.5% Triton X-100 in 1× PBS for 30 min. Plates were incubated with rhodamine-conjugated phalloidin (6 nM) for 1 h with rocking at room temperature followed by Hoescht dye for 2 min. Glass coverslips were mounted on the plate surface, and staining was observed by epifluorescence microscopy. Color overlay images were created using Metamorph software. Images were captured at 100× magnification.

RESULTS

While the differentiation of Rcho-1 cells into the invasive, giant cell phenotype has been well documented, this study sought to determine if the Rcho-1 cell line represents an exclusive trophoblast giant cell population and to determine if hypoxia has an inhibitory effect on trophoblast giant cell differentiation. To answer these questions, our laboratory developed an RT-PCR Trophoblast Gene Expression Profile and examined Rcho-1 cells for expression of trophoblast lineage-specific genes. Using genes that have been identified previously by in situ hybridization or immunolocalization as trophoblast lineage-specific markers (Table 1), primers targeted to each gene were used to examine Rcho-1 mRNA expression (Table 2). Cells were harvested at various time points throughout the differentiation process. First, the effect of differentiation on the expression of the stem cell marker *Id2* was analyzed by semiquantitative RT-PCR. *Id2* expression is associated with undifferentiated, proliferative, trophoblast stem cells. Differentiation of Rcho-1 cells for 7 days downregulated the expression of *Id2* mRNA (Fig. 3).

The expression of trophoblast giant cell-specific genes, *Snai1*, *Hand1*, and *Csh1*, was also examined by RT-PCR in Rcho-1 cells (Fig. 4). *Hand1* and *Snai1* mRNA were detected in undifferentiated Rcho-1 cells, whereas *Csh1* expression was not. On differentiation, the semiquantitative RT-PCR analysis indicated that *Hand1* expression was maintained, *Snai1* expression was increased, and *Csh1* expression was upregulated. The spongiotrophoblast marker (*Tpbpa*) as well as the labyrinthine markers (*Esx1* and *Tec*) were not detected in undifferentiated or differentiated Rcho-1 cells, as predicted (Fig. 5). Primers specific for beta-actin mRNA were used as internal controls for lineage marker analysis, except in the case of *Esx1*, where inclusion of actin primers interfered with results. While the expression of an additional labyrinthine marker, *Tcfeb*, was detected in Rcho-1 cells (data not shown), this is likely due to their transformed state as a choriocarcinoma [73]. As expected, spongiotrophoblast and labyrinthine markers were detected in differentiated TS_{TS} cells [68]. Because this study also examined the effect of hypoxia on the differentiation of Rcho-1 cells, lineage-specific expression was analyzed when cells were cultured in hypoxia at 1% oxygen. Lineage-specific gene expression in undifferentiated and differentiated Rcho-1 cells cultured in hypoxia for 7 days

![FIG. 3. Differentiation downregulates trophoblast stem cell marker *Id2* in Rcho-1 cells. *Id2* (497 bp) expression was analyzed in undifferentiated (U, Day 7) and differentiated (D, Day 7) Rcho-1 cells by RT-PCR. Primers specific for beta-actin (243 bp) were used as internal controls for reactions. M indicates 100-bp DNA molecular length marker.](image)

![FIG. 4. Rcho-1 cells express lineage-specific trophoblast giant cell markers. RT-PCR analysis of *Snai1* (496 bp), *Hand1* (793 bp), and *Csh1* (746 bp) in undifferentiated (U, Day 7) and differentiated (D, Day 7) Rcho-1 cells. Primers specific for beta-actin (243 bp) were used as internal controls for reactions. M indicates 100-bp DNA molecular length marker.](image)

![FIG. 5. Rcho-1 cells do not express lineage-specific spongiotrophoblast or labyrinthine markers. *Tpbpa* (458 bp), *Esx1* (254 bp), and *Tec* (663 bp) expression in undifferentiated (U, Day 0) and differentiated (D, Day 7) Rcho-1 cells was examined by RT-PCR. Differentiated TS_{TS} cells (T) were used as positive controls for spongiotrophoblast (*Tpbpa*) and labyrinthine (*Esx1*, *Tec*) expression. Primers specific for beta-actin (243 bp) were used as internal controls for reactions except in the analysis of *Esx1*, where inclusion of actin primers interfered with results. M indicates 100-bp DNA molecular length marker.](image)

![FIG. 6. Hypoxia prevents downregulation of trophoblast stem cell marker *Id2* in differentiating Rcho-1 cells. *Id2* (497 bp) expression in Rcho-1 cells cultured in differentiation media and exposed to normoxia (N, 21% oxygen) or hypoxia (H, 1% oxygen) was examined by RT-PCR. Day of differentiation is indicated. Primers specific for beta-actin (243 bp) were used as internal controls for reactions. M indicates 100-bp DNA molecular length marker.](image)
was analyzed by RT-PCR. Rcho-1 cells cultured in differentiation media in 1% hypoxia maintained the expression of the stem cell marker *Id2* (Fig. 6). In addition, Rcho-1 cells differentiated in hypoxia for 7 days retained their trophoblast giant cell-specific gene expression profile of *Snail1*, *Hand1*, and *Csh1* (Fig. 7). Spongiotrophoblast and labyrinthine markers *Tpbpa*, *Esx1*, and *Tec* were not expressed by Rcho-1 cells under hypoxic conditions (Fig. 8). These results confirm that the Rcho-1 cell line represents a stem cell-like population that expresses exclusive markers of the trophoblast giant cell lineage and that culturing Rcho-1 cells under hypoxic conditions does not alter their lineage commitment.

The invasive trophoblast giant cell phenotype is also associated with the expression of several genes, including *Stra13*, *Mmp9*, alpha-5 integrin, and alpha-1 integrin. Our data indicate that expression of these genes can be detected by RT-PCR in differentiated Rcho-1 cells cultured for 7 days in normoxia (Fig. 9). Exposing Rcho-1 cells to hypoxia for 7 days did not prevent the expression of *Stra13*, *Mmp9*, alpha-5 integrin, or alpha-1 integrin (Fig. 9). Again, primers specific for beta-actin mRNA were used as internal controls, except in the cases where inclusion of actin primers interfered with results.

To examine the effect of hypoxia on a functional aspect of trophoblast giant cell differentiation, CSH1 protein induction was analyzed by Western blot, using polyclonal antibodies (Fig. 10). On differentiation, Rcho-1 cells secrete CSH1 (∼40 kDa), and our results indicate that differentiation induced CSH1 protein levels in Rcho-1 whole cell lysates. Culturing in hypoxia, however, abolished this induction. A hypoxia-insensitive, cross-reactive band acted as a fortuitous loading control.

The effect of hypoxia on the differentiation of Rcho-1 cells was also examined by analyzing changes in cell morphology. Differentiating Rcho-1 cells exhibit dramatic changes in morphology, becoming enlarged in size and multinucleate. To more clearly identify alterations in cell size and shape,
lineage. In the undifferentiated state, Rcho-1 cells expressed the trophoblast stem cell marker Id2, and when Rcho-1 cells were differentiated in normoxia, they downregulated Id2 expression and continued expression of trophoblast giant cell markers. In hypoxic conditions, Rcho-1 cells cultured in differentiation media also expressed trophoblast giant cell markers; however, downregulation of the stem cell marker Id2 was inhibited. Hypoxia’s prevention of CSH1 protein induction indicated an inhibition of differentiation on the functional level, while a morphological alteration was also evident with the loss in stress fiber formation in hypoxia. Hypoxia also prohibited the induction of palladin protein, an essential mediator of stress fiber assembly [67].

In addition, to determine if hypoxia/reperfusion may play a role in the inhibition of trophoblast giant cell differentiation, Rcho-1 cells were initially differentiated in hypoxia and then re-exposed to normoxic conditions. Cells that had undergone this normoxic switch were able to differentiate based on the formation of stress fibers and the induction of palladin protein (data not shown). Thus, hypoxia’s inhibition of trophoblast giant cell differentiation was reversible on reperfusion in conditions of 21% oxygen. These results demonstrate that hypoxia inhibits aspects of Rcho-1 trophoblast giant cell differentiation at the morphological, functional, and molecular levels.

**DISCUSSION**

Many studies of placental development and trophoblast differentiation have set the stage for the current investigations into the molecular mediators of trophoblast differentiation. Previous reports examining placental explants, isolated primary trophoblast cells from early and term pregnancies, and choriocarcinoma-derived cell lines have characterized the various phenotypes of trophoblast subtypes as well as investigated the effect of a multitude of growth factors and extracellular matrix on trophoblast differentiation. Several transcriptional regulators of trophoblast lineage commitment have also been identified [1, 11, 13]. In many of these studies, the isolation of trophoblasts was confirmed by expression of epithelial markers, such as cytokeratin 7, while lineage specificity of the trophoblasts examined was determined by the phenotypic activity (i.e., secretion, fusion, invasion, etc.) of the cells and not necessarily by analysis of lineage-specific gene expression. While these studies undoubtedly did produce trophoblast subtypes, the isolation of specific trophoblast lineages has proven to be difficult, and the exclusion of other lineages was not always performed, affecting the possible interpretation of results.

Since several defects in placental development are attributed to alterations in specific trophoblast lineages, the necessity for isolated trophoblast lineage models is becoming necessary to delineate the molecular mechanisms involved in placental pathologies. For example, preeclampsia is associated with defects in the differentiation of invasive trophoblast cells. While several studies have shed light on the differentiation process of human undifferentiated trophoblasts to the invasive phenotype, we sought an isolated trophoblast model, free from the possible interference of other trophoblast subtypes, in which to examine the differentiation of invasive trophoblast cells. The Rcho-1 cell line has been documented to exhibit an invasive, trophoblast giant cell phenotype, and although it was derived from an induced choriocarcinoma, it exhibits a stepwise differentiation that parallels giant cell differentiation in vivo. In order to determine if the Rcho-1 cell line represents an isolated trophoblast lineage, we examined Rcho-1 gene expression using an RT-PCR Trophoblast Gene Expression...
HYPOXIA INHIBITS GIANT CELL DIFFERENTIATION

Profile developed by our laboratory. The markers examined in our profile have been documented by in vivo studies to be genes expressed exclusively by specific trophoblast lineages. Importantly, several of these lineage-specific markers are highly conserved between human and rodent, and many have been implicated as essential for the differentiation of trophoblast lineages.

Using semiquantitative RT-PCR, our data demonstrate that both undifferentiated and differentiated Rcho-1 cells express the giant cell markers Snail and Hand1 and that the expression of Snail and Csh1 is upregulated on differentiation. While undifferentiated Rcho-1 cells maintained the capacity to proliferate and expressed the stem cell marker Id2, their stem cell-like state did not include the expression of the spongiotrophoblast marker or the labyrinthine markers Esg1 and Tec. Surprisingly, the expression of Tcfeb, another labyrinthine marker, was detected in Rcho-1 cells. This aberrant expression, however, is unlikely to indicate pluripotency and is more probably a result of their transformed state as a choriocarcinoma since upregulated expression of Tcfeb has also been documented in papillary renal carcinomas due to a chromosomal translocation [73]. In any case, differentiation of Rcho-1 cells did not stimulate expression of the spongiotrophoblast markers or the other labyrinthine markers, indicating that differentiation of Rcho-1 cells does not result in a mixed lineage population. Additionally, differentiation of giant cells at the molecular level is characterized by decreased expression of the transcription factor Id2, and decreased Id2 expression was observed in differentiating Rcho-1 cells.

The use of the Trophoblast Gene Expression Profile has allowed us to definitively classify the Rcho-1 cell line as an isolated, stem cell-like trophoblast population committed to the giant cell lineage. Our results place undifferentiated Rcho-1 cells at a point between trophoblast stem cells and differentiated giant cells, while differentiated Rcho-1 cells exhibit both the genetic profile and the phenotypic characteristics of trophoblast giant cells (Fig. 13). This allows for unambiguous interpretation of the results of this and previous studies examining the regulation of Rcho-1 giant cell differentiation. Genetic profiling of other model systems will provide invaluable information regarding trophoblast lineage commitment as well as the mediators and conditions affecting trophoblast differentiation.

One of the conditions that has previously been shown to alter trophoblast differentiation is hypoxia. Studies using placental explants (which represent a mixed trophoblast population) or undifferentiated trophoblasts (which have the potential to adopt multiple phenotypes) have demonstrated that hypoxia inhibits differentiation of the invasive trophoblast phenotype [14, 37]. Hypoxia, in concert with other environmental factors, induces proliferation in trophoblast stem cells. Thus, hypoxia stimulates the rapid growth needed to amass the quantity of cells required for formation of the placenta during the early phase of pregnancy in which the size of the placenta far exceeds the size of the developing embryo [49]. During the initial rounds of DNA replication, hypoxia also likely prevents DNA damage from oxidative metabolites [74]. Subsequently, the variation in oxygen exposure that trophoblast giant cells encounter in their migration and invasion into the maternal tissues suggests that changes in oxygen concentrations could act as a regulator of trophoblast cell differentiation, perhaps indicating positive interaction with the maternal environment or successful advancement to their destination. Thus, increasing oxygen availability could act as a signal in the stepwise differentiation process, while extended hypoxia would not be the preferred physiological condition and could lead to placental defects.

In this study, differentiation of Rcho-1 cells cultured under hypoxic conditions demonstrated that low oxygen levels did not alter the expression of giant cell lineage markers, particularly a known mediator of giant cell lineage commitment, Hand1. Nor did hypoxia induce expression of labyrinthine or spongiotrophoblast markers. Thus, any alteration in differentiation in hypoxia that is observed in Rcho-1 cells was not due to a shift in lineage commitment. Furthermore, hypoxia also did not prevent the expression of Stra13, Mmp9, alpha-5 integrin, and alpha-1 integrin, genes conserved in invasive trophoblasts in humans and rodents, supporting their continued commitment to the invasive lineage. In contrast, our data also showed sustained Id2 expression in Rcho-1 cells cultured in hypoxia. These results concur with previous studies showing that Id2 expression is upregulated in trophoblasts cultured in hypoxia [14, 50]. Whereas the lineage markers mediating trophoblast giant cell differentiation may be constitutively expressed in Rcho-1 cells, the continued expression of Id2 appears to perpetuate a stem cell-like state when Rcho-1 cells are cultured in hypoxia. A previous report demonstrated that Id2 overexpression inhibits invasive trophoblast differentiation [14]. Whether continued expression of endogenous Id2 in Rcho-1 cells could act alone or in collaboration with other mediators to inhibit Rcho-1 giant cell differentiation is under investigation. Additionally, how hypoxia upregulates Id2 is not clear. One possibility is the stabilization of the relatively unstable Id2 mRNA [75]; however, a more likely possibility is increased transcription mediated by the oxygen-regulated Hypoxia Inducible Factor1 (HIF1) [76]. Initial studies by Lofstedt et al. [77] that examined hypoxic regulation of the Id2 promoter implicate HIF1 as a possible transactivator. The mechanisms that increase Id2 expression in hypoxia in trophoblast cells are currently under investigation.

Our data also indicate that several other aspects of giant cell differentiation were inhibited by hypoxia. Like primary trophoblast cells, differentiated Rcho-1 giant cells secrete the
placental lactogen family of hormones, including CSH1. While hypoxia did not inhibit expression of Csh1 mRNA, low oxygen conditions did prevent detection of CSH1 protein (secreted weight ~40 kDa) in whole cell lysates. While peptide hormone secretion in the placenta is typically constitutive, hypoxia could be acting as a previously unidentified master regulator of CSH1 protein synthesis. This level of regulation could be similar to other systems in which hormone expression has been demonstrated to be constitutive, while secretion is controlled by signaling events or other mechanisms [78, 79]. Whether induction of CSH1 protein levels in Rcho-1 cells was hindered at the stage of CSH1 protein synthesis or protein processing is not clear at this time. In contrast, another possibility for the loss of detectable CSH1 protein at the secreted weight in whole cell lysates could be hypoxia-induced hypersecretion of the protein. If this were the case, however, the constitutive nature of peptide hormone expression and processing in the placenta would suggest that at least low levels of the secreted weight, protein would be detectable in whole cell lysates.

Trophoblast giant cell differentiation causes dramatic increases in cell size and the formation of multinucleate cells. This alteration in size is accompanied by the formation of actin stress fibers [66, 67]. Under light microscopy, the morphological differences between Rcho-1 cells cultured in differentiation media in normoxia and those cultured in differentiation media in hypoxia were subtle. Cells in both conditions showed increases in size. The enlargement in cell size in hypoxia, initially thought to be part of the differentiation process, may be due to an inherent cellular response to low oxygen, as an increase in cell surface could allow for an increase in the rate of oxygen diffusion. Staining with F-actin-binding phallolidin, however, illustrated a dramatic difference in stress fiber formation. Although the hypoxic cells enlarged in size, the cytoskeletal organization was clearly altered. Previous studies have demonstrated that hypoxia has differential effects on the formation of stress fibers depending on cell type and activation of intracellular signaling pathways [80–83]. In our study, this dramatic morphological change was evident in Rcho-1 cells differentiated in normoxia but was blocked by hypoxia. The perturbation of stress fiber assembly, however, leads to possible alterations in cell motility and extracellular matrix signaling complexes, both of which have roles in giant cell differentiation. Additionally, since stress fibers insert into the membrane at focal adhesions, sites where extracellular signals are transmitted via intracellular signaling pathways, the loss of stress fiber formation indicates a significant change in the intracellular signaling complexes downstream of the integrins. This alteration modifies the ability of Rcho-1 cells to integrate ECM signals, which have been demonstrated to regulate giant cell differentiation [84]. The molecular mechanism mediating hypoxia’s inhibition of stress fiber development in Rcho-1 cells is under investigation.

The formation of stress fibers involves a complex array of proteins, including F-actin, alpha-actinin, and protein tyrosine kinase 2 (previously named focal adhesion kinase); yet work by Parast and Otey [66] identified a regulator of the process in trophoblast cells, the recently identified palladin protein. Palladin is an actin-associated phosphoprotein that binds alpha-actinin and members of the vasodilator-stimulated phosphoprotein family, which are localized to stress fibers [66]. While palladin expression in the placenta has not been characterized, its expression is essential for embryonic viability, regulated during development, and downregulated in mature adult tissues [66, 85]. This modulation in expression implicates palladin in the cytoskeletal rearrangements necessary for cellular differentiation. Specifically, loss of palladin expression in Rcho-1 cells and fibroblasts blocked stress fiber formation [66]. In our studies, hypoxia completely blocked the induction of palladin protein, suggesting that palladin is a mediator of hypoxia’s inhibition of morphological differentiation. Hypoxia’s inhibition of palladin protein could occur at the level of protein synthesis or processing, as appears to be the case with CSH1 protein induction, or hypoxia could be modulating the transcription of the palladin gene, implicating transcriptional regulators such ID2 or HIF1 [14, 76].

The cellular responses to low oxygen conditions are mediated in large part by the transcriptional activator HIF1 [76]. While several reports underscore the possible role of HIF1 in the development of preeclampsia and in the inhibition of the invasion process, preeclampsia is associated primarily with defects in the differentiation of the invasive trophoblast lineage specifically, making the Rcho-1 cell line an ideal model for investigations into the molecular mediators controlling hypoxia’s inhibition of trophoblast giant cell differentiation [37, 86].

In conclusion, our studies have determined that Rcho-1 cells can be maintained as a proliferative, lineage-specific cell line that is committed to only the trophoblast giant cell lineage on differentiation. In addition, our studies indicate that hypoxia inhibits aspects of the molecular (Id2 expression, palladin protein induction), morphological (stress fiber formation), and functional (CSH1 protein induction) differentiation of Rho-1 giant cell formation. The multifaceted effects of hypoxia on giant cell differentiation in this model also lead to additional questions regarding hypoxic modulation of protein synthesis and processing as well as hypoxic regulation of extracellular matrix signaling proteins. Future analysis of the effects of hypoxia-sensitive regulators, such as HIF1, on the differentiation of lineage-specific trophoblast models will provide further insight into the possible causes of placental pathologies.

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