Hypoxic Induction and the Role Of HIFS in the Activation of Luciferase Constitutive Reporters in Placental Stem Cells

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HYPOXIC INDUCTION AND THE ROLE OF HIFs IN THE ACTIVATION
OF LUCIFERASE CONSTITUTIVE REPORTERS
IN PLACENTAL STEM CELLS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

DIANE MICHELLE DORAN
B.A., Lake Forest College, 2004

2007
Wright State University
WRIGHT STATE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

August 29, 2007

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Diane Michelle Doran ENTITLED Hypoxic Induction and the Role of HIFs in the Activation of Luciferase Constitutive Reporters in Placental Stem Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Hypoxia is critically important to the development of the embryo and placenta. Proper placental development is critical for normal fetal growth and embryonic survival. Abnormal placental development has been implicated in numerous obstetric complications, including preeclampsia, which affects about 7% of all pregnancies and can be fatal for both mother and baby.

Rodent and murine trophoblast stem cells differentiate into three distinct cell lineages: giant cells, spongiotrophoblasts, and labyrinthine cells, which form different layers and have different functions within the placenta. Recent studies in our laboratory have focused on the invasive giant cell layer using the rodent Rcho-1 choriocarcinoma stem cell-like cell line, which has been shown to be committed to differentiate into the giant cell lineage.

It has been shown that chronic exposure to hypoxia inhibits Rcho-1 trophoblast differentiation. One factor that has been extensively studied in the field of hypoxia are Hypoxia-Inducible Factors (HIFs), conserved, heterodimeric proteins that bind to DNA under hypoxic conditions to up regulate oxygen-dependent gene transcription. Whereas HIF-β (ARNT) is ubiquitously expressed and stable, HIF-α is subject to rapid turnover in normoxia and has been the major focus in studies examining HIF regulation. Although
HIF-1α has been widely studied, few studies have been done on the function of HIF-2α in the regulation of trophoblast differentiation.

Studies in our laboratory sought to characterize the mechanism of giant cell differentiation and placental formation and have recently focused on HIF-1α. However, it is possible that HIF-1α and HIF-2α play complementary roles in controlling trophoblast differentiation and placental formation \textit{in vivo}, therefore this study examined the role of HIF-2α in Rcho-1 trophoblast differentiation. Previous studies examined the transcriptional activity of HIF-1α in response to hypoxia in the Rcho-1 trophoblast placental stem cell line, using a conventional luciferase dual-reporter assay. However, it was consistently observed that the level of activation of the constitutive reporter was significantly higher in hypoxic samples than in normoxic controls; therefore, this study also examined the role of HIF-1α in the unexpected induction of luciferase constitutive reporters after exposure to hypoxia, using a more reliable transfection method.

The results of this study indicate that HIF-2α protein is not detectable in Rcho-1 cells, even after exposure to hypoxia. This study also found that the hypoxic induction of luciferase constitutive reporters was a phenomenon independent of species or cell-type and sequence analysis revealed that hypoxic induction of the luciferase constitutive reporters was independent of HIF-1α. Sequence analysis also revealed the consensus sequences for several other transcription factor binding sites, including steroid hormones and NF-κB. Finally, this study demonstrates a more reliable method of controlling for transfection efficiency that negates the need for luciferase constitutive reporters, avoiding potential error caused by their hypoxic induction.
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INTRODUCTION

A. Hypoxia and Placental Development

Embryonic implantation and placental formation occur under severe hypoxic conditions (~3% O₂) [1-9]. Measures of oxygen tension between eight to ten weeks of pregnancy indicate that the partial pressure of oxygen is significantly lower in the placenta than in the surrounding endometrium (~14% O₂) [10]. Furthermore, oxygen tension slowly increases until about week 13 of pregnancy, when the partial pressure of oxygen is approximately equal in the placenta and the endometrium [10]. Hypoxia is important for the protection of the very early embryo from oxidative stress as the placenta does not begin to produce superoxide dismutases until approximately the eighth week of gestation [10]. Exposure to oxygen before this time would expose the fetus to oxidative damage, possibly resulting in birth defects or spontaneous abortion. The formation of the placenta from the trophoblasts occurs in a manner which protects the fetus from this threat.

Placental development involves the interaction of fetal and maternal tissues in an extremely complex process. In the murine blastocyst, the first two cell lineages to develop are the trophoblast and the inner cell mass (ICM) (Figure 1). The trophoblast surrounds the ICM, which becomes the fetus and has different functions based on its relationship to the ICM [11-13]. The mural trophoblast lies opposite, and does not contact, the ICM. This portion of the trophoblast differentiates into primary giant cells which invade the maternal decidua as the site of initial attachment of the
Figure 1: **Origin of trophoblast stem cells.**

The first two cell lineages that arise from the murine blastocyst are the trophectoderm and the inner cell mass (ICM). The ICM becomes the embryo. The mural trophectoderm differentiates into primary giant cells which invade the maternal decidua to anchor the blastocyst. Trophoblast stem cells arise from the polar trophectoderm of the embryo, just above the inner cell mass. Figure modified from Gultice AD, Wright State University, 2005.
Polar Trophectoderm

Inner Cell Mass (ICM)

Blastocyst

Mural Trophectoderm
blastocyst to the uterine lining [12-14]. Placental stem cells arise from the polar
trophectoderm of the embryo, just above the inner cell mass. After the embryo attaches
to the endometrial wall, trophoblast stem cells from the polar trophectoderm begin to
invade the maternal uterine tissue [12, 15]. The area of intimate contact between the
fetal and maternal tissues is known as the junctional zone [15]. The junctional zone is
further divided into regions, based on proximity to fetal or maternal tissues. The basal
plate lies on the embryo/fetal side of the junctional zone and the placental bed lies on the
maternal side; all trophoblast cells residing outside the placental villi are termed
extravillious trophoblasts in humans or trophoblast giant cells in rodents [15]. These
trophoblasts then differentiate to distinct lineages that represent different functional
layers within the placenta.

Proper placental development is critical for normal fetal growth and embryonic
survival [4-6]. Its invasion and attachment in the uterus serves as the maternal-fetal
interface allowing the secretion of hormones necessary to maintain pregnancy, while
providing a barrier against maternal immunological attack and mediating the exchange of
nutrients and wastes between mother and child [4-6, 16-19]. Irregular or abnormal
placental development has been implicated in numerous complications of pregnancy
including intrauterine growth retardation, spontaneous abortion, and maternal
hypertension, which can lead to a potentially fatal condition known as preeclampsia [4-6,
15, 20].
B. Studying Trophoblast Differentiation

Cell Line Models

The placenta in rodents and humans is similar in many ways and is composed of analogous cell types which display similar functions. Additionally, there is some conservation between transcription factors necessary for the expression of several placental genes and the trophoblast cell lineages appear to follow the same pathways despite overall differences in the morphogenesis of rodent and human placentas [11, 16, 19]. Rodent and murine trophoblast stem cells differentiate into three distinct cell lineages: giant cells, spongiotrophoblasts, and labyrinthine cells (Figure 2) [11, 16-17, 21]. These cell types express conserved genes and mediate placental formation and are similar in function to human cell types [11, 16-19]. Due to the difficulty of studying human placental development in vivo and of obtaining human stem cells, several rodent and murine cell lines have been developed to study placental trophoblasts in vitro. Previous studies in our laboratory developed a lineage profile for each of the different cell types found in vivo and used these profiles to characterize each cell line [22].

The TS\textsubscript{3.5} cell line, isolated from a mouse blastocyst at day 3.5 of gestation, is a trophoblast stem cell line that can be induced to differentiate [6, 13, 22]. Lineage analysis has shown that TS\textsubscript{3.5} cells have the ability to differentiate into all three murine cell types simultaneously, specifically, labyrinthine cells, spongiotrophoblasts, and giant cells [13, 17, 22]. Studies have indicated that TS\textsubscript{3.5} cells can be induced to differentiate toward the giant cell lineage depending on the treatment used to induce differentiation, however, other cell lineages may still be present in the culture making the exclusive study of the giant cell lineage difficult [21-23].
Figure 2: Trophoblast stem cell lineages and functions

Rodent and murine trophoblast stem cells differentiate into three lineages with distinct morphologies and functions. The labyrinthine layer is responsible for the transport of wastes and nutrients across the placenta. The spongiotrophoblast layer serves as a barrier, with no other known function while the giant cell layer is the invasive layer, responsible for invading the maternal decidua and establishing blood flow to the developing embryo. Analogous cell types have been identified in humans for the labyrinthine and giant cell layers. Figure modified from Gultice et al. Biol. Reprod. 2006; 74(6): 1041-1050.
Proliferative Trophoblast Stem Cells

- Labyrinthine
  - Transport
- Spongiotrophoblast
  - Barrier
- Giant cell
  - Invasion
The SM-10 cell line is another mouse trophoblast stem cell-like cell line and has recently been reported to be committed to differentiate into the labyrinthine lineage [22, 24-25]. The labyrinthine lineage is composed of cells responsible for the transport of nutrients, wastes, and other materials between mother and fetus. It is one of the two cell types analogous to the human placenta, where it is called the syncytiotrophoblast, and the lineage analysis protocol designed by our lab revealed that it does differentiate exclusively toward the labyrinthine lineage in vitro when treated with TGF-β [16, 22, 24].

The second analogous cell type is the rodent giant cell lineage, which is responsible for the invasion of the uterine lining and remodeling of the maternal vasculature in order to deliver blood supply to the developing embryo [6, 11, 16, 21, 24]. A cell line that serves as a model for this lineage is the rat Rcho-1 choriocarcinoma stem cell-like cell line. The Rcho-1 cell line, developed by Dr. Michael Soares, serves as a model of the invasive trophoblast giant cell placental lineage and has been used extensively [6, 22, 26]. Lineage analysis studies have characterized this cell line on the molecular, morphological, and functional levels and determined that the Rcho-1 trophoblast stem cell line is committed to differentiate only into the invasive, giant cell phenotype (Figure 3). This allows studies using this cell line to focus solely on the function and importance of the giant cell placental layer.

c. Regulation of Giant Cell Differentiation

*Hypoxia-Inducible Factors*

Increasing levels of oxygen tension regulate the timing of trophoblast giant cell migration, invasion and differentiation into the maternal decidua and the remodeling of
Figure 3: Rcho-1 Trophoblats

Rcho-1 trophoblats are a rodent choriocarcinoma derived stem cell-like cell line that model the invasive giant cell lineage. When differentiated, they exhibit the typical morphological, functional, and molecular changes characteristic of giant cells including decreased expression of Inhibitor of differentiation 2, increased expression of palladin and chorionic somatomammotropin hormone-1 (CSH-1), increased actin stress fiber formation, endoreduplication, and a dramatic increase in cell size [6, 22, 26]. Cells are shown magnified 400x. A. Rcho-1 stem cells. B. Rcho-1 differentiated cell.
the maternal vasculature, though no study has conclusively determined the exact mechanisms for these events [4-6, 20, 27]. It has been shown, however, that chronic exposure to hypoxia inhibits trophoblast giant cell differentiation, suggesting that a failure in migration or invasion may be enough to stall differentiation and cause placental complications [4-6, 20, 27].

One factor that has been extensively studied in the field of hypoxia is a family of oxygen-sensitive, basic helix-loop-helix transcription factors known as hypoxia-inducible factors, or HIFs. HIFs are highly conserved, heterodimeric proteins that bind to DNA under hypoxic conditions to up-regulate oxygen-dependent gene transcription [1-4, 6-9, 12, 27-55]. The functional heterodimer consists of a phosphorylation-dependent HIF-α subunit and a constitutively expressed HIF-β subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). To date, studies have identified three HIF-α homologs named HIF-1α, HIF-2α, and HIF-3α, though most studies focus on HIF-1α or HIF-2α (also identified as Endothelial PAS1, HIF-related factor, HIF-like factor, and MOP2) [2-4, 8-9, 28, 34-36, 38, 61-64]. Whereas HIF-1α mRNA expression is present in most cell types, HIF-2α mRNA expression is more cell-type specific, appearing predominantly in epithelial, neuronal, and fibroblast tissue as well as many tumors [3, 32-33, 35]. HIF-2α has been shown to have about 48% sequence homology to HIF-1 with highly conserved bHLH, PAS and oxygen degradation-dependent (ODD) domains suggesting that the two proteins have identical regulatory mechanisms [4, 32-33, 36]. Although HIF-1α and HIF-2α are known to function as heterodimeric transcription factors, the function of HIF-3α is still unclear. However, a short splice variant of HIF-3α,
inhibitory PAS protein (IPAS), has been shown to function as a transcriptional repressor [2].

Knockout studies indicate that, despite their similarity, HIF-1α and HIF-2α have some non-redundant functions. Studies using placental stem cells from HIF-1α−/−, HIF-2α−/−, and ARNT−/− mice reveal that knockout of only one HIF-α subunit results in a much less severe placental defect than seen in a double knockout, indicating that some overlap in function does exist. However, HIF-1α−/− mice exhibit placental blood vessel defects while HIF-2α−/− mice exhibit abnormal lung develop and vascular defects [3, 33]. Knockdown of both HIF-α subunits together results in severe placental defects that mimic those seen in ARNT−/− mice. In all cases, HIF-1α−/− and ARNT−/− knockouts are embryonic lethal by day 10.5 [7, 13]. HIF-1α−/− and ARNT−/− also change the fate of placental differentiation, shifting trophoblast cell fate toward less invasive cells types, resulting in shallow invasion of the placenta into the maternal decidua and poor fetal vascularization of the placenta. HIF-1α−/− and ARNT−/− knockout in TS 3.5 cells shifts differentiation away from the invasive giant cell phenotype toward the less invasive labyrinthine lineage [4, 7, 13]. Despite the apparent non-redundancy of function between HIF-1α and HIF-2α and its cell-type specificity, few studies have been done to elucidate the functional role of HIF-2α in trophoblast differentiation or its role in placental formation independent of HIF-1α. It has been reported, however, that HIF-2α is present in vascular endothelial cells within the placenta [7, 33, 35, 37].

HIF-α subunits are continually expressed in most human tissues but are very rapidly degraded under normal oxygen and metabolic conditions through the ubiquitin-dependent proteasomal degradation pathway [2-9, 7, 9, 12, 28-32, 43-47, 51-53, 55]. The
Figure 4: HIF-α subunit

HIF-1α and HIF-2α are 48% conserved overall but are nearly identical in the Per ARNT Sim (PAS) domain, oxygen-dependent degradation domain, and the transactivation (TA) domains. The HIF-α subunit is represented graphically along with the conserved amino acids that are hydroxylated to regulate its stability and activity, two prolines (P) in the oxygen-dependent degradation domain (ODD) and an asparagine (N) in the C-terminal transactivation domain (CTAD). Figure modified from Wenger, et al, Sci. STKE, 2005.
HIF-β heterodimer partner is ubiquitously expressed and stable in most cell types [3, 29, 38, 40, 45, 51-53]. The HIF-α and β subunits must dimerize in order for the HIF protein to be functionally active. Whereas HIF-β is constitutively expressed and not subject to hypoxic regulation, HIF-α contains several functional domains, including the oxygen-dependent degradation domain (ODD) and the C-terminal transactivation domain (CTAD), whose modification allows for the hypoxic regulation of the protein (Figure 4) [2-3, 36, 38, 40, 43, 51-52, 70]. Under normal oxygen conditions, key proline and asparagine residues within the ODD and CTAD of the α-subunit are hydroxylated by prolyl and asparaginyl hydroxylases (PHD2 and Factor-Inhibiting HIF (FIH), respectively), targeting HIF-α for ubiquitination and degradation through interactions with the von Hippel-Lindau tumor suppressor protein (VHL) [2, 4, 9, 32, 36-39, 47-49, 51-53]. Prolyl hydroxylases require both Fe(II) and dioxygen as cofactors. If one of these cofactors is removed, the enzyme is unable to act, allowing HIF-α to escape degradation. Therefore, during hypoxic conditions, hydroxylation and ubiquitination are blocked due to the lack of available oxygen, causing the HIF-α subunit to stabilize and allowing it to dimerize with the HIF-β subunit and recruit co-factors, such as p300, to become transcriptionally active (Figure 5) [2-4, 12, 29-30, 36, 39, 43, 48, 51-52, 55]. Interestingly, VHL−/- and PHD2−/- mice, which have an abundance of HIF-α, are also embryonic lethal due largely to defects in the placenta [9, 12, 50, 56]. Similar to results seen with HIF-1α and ARNT knockdown, PHD2−/-, which increases HIF-α levels, also shifts TS cell differentiation toward the labyrinthine lineage [4, 7, 9, 12]. Additionally, increased levels of HIF-1α and HIF-2α protein expression have been observed in tissue samples from pre-eclamptic placentas [4, 20]. This paradox underscores the complexity
Figure 5: HIF-α subunit degradation and stabilization.

Under normal oxygen conditions, the HIF-α subunit is hydroxylated at conserved proline and asparagine residues in the oxygen-dependent degradation domain and C-terminal transactivation domain by PHD2 and FIH. The hydroxylation targets HIF-α for ubiquitination by VHL. Polyubiquitination then targets HIF-α for degradation in the proteasome. Low oxygen levels inhibit the action of PHD2 and FIH, allowing the HIF-α subunit to escape degradation. It is then able to dimerize with HIF-β (ARNT), recruit cofactors such as p300/CBP, and bind to HREs in the DNA, activating hypoxia-responsive genes. Figure was modified from Bhattacharya and Ratcliffe, Nat. Struct. Biol., 2003, 10(7): 501-503.
HIF-α

PHD₂ & FIH
Normoxia

HIF-α

OH OH OH
VHL

Ub Ub Ub
Proteasome

HIF-α

Hypoxia

HIF-β

HIF-α

p300/CBP

HRE

Angiogenesis
Vascularization
Glucose metabolism
of placental development and indicates that proper regulation of HIF levels is critical for correct development of the placenta [12].

The functional HIF protein binds to Hypoxia Response Elements (HREs) located within the promoter regions of genes necessary for cell survival under low oxygen conditions, including genes involved in angiogenesis and glycolysis such as erythropoietin (EPO), phosphoglycerate kinase-1 (PGK), and vascular endothelial growth factor [3, 5-6, 28-29, 33-34, 50, 52-55, 57-60]. Because a number of cofactors are necessary for HIFs to be transcriptionally active, including p300/CBP, when working with HIFs it is important to assay for transcriptional activity as well as the presence of protein [2, 44, 51]. For example, in 2003, Hu et al reported that though HIF-2α protein had been observed in murine ES and mouse embryonic fibroblasts and was able to heterodimerize with HIF-β and bind to HREs, the protein was nonfunctional as a transcription factor due to binding of a repressor protein [33]. Western blotting, which can estimate relative amounts of stable protein in cell extracts, cannot be used alone to analyze the activity of HIFs. Therefore, a more direct measure must be used to analyze the effects of treatment on HIF activity. One of the easiest ways this can be accomplished is through luciferase reporter assays, which measure the activity of luciferase reporter genes under the control of a target promoter to represent the up-regulation of HIF target genes as an indication of HIF transcriptional activity.

**D. Previous Studies**

Studies in our laboratory sought to characterize the mechanism of giant cell differentiation and placental formation. Previous studies in our lab and others have
determined that Rcho-1 cells are committed to differentiating solely into trophoblast giant
cells and can serve as a model cell line for this process. Further, studies in our laboratory
identified hallmarks of giant cell differentiation on the molecular, morphological, and
function levels, providing concrete endpoints for the study of differentiation [6, 22].
These five endpoints are 1) decreased expression of Inhibitor of Differentiation-2 (Id-2),
2) induction of palladin protein, 3) induction of chorionic somatomammotropin
hormone-1 (CSH-1), 4) a dramatic increase in cell size, and 5) the rearrangement of the
actin cytoskeleton [6].

Using these endpoints, Gultice et al investigated the effect of hypoxia on Rcho-1
giant cell trophoblast differentiation in vitro [6]. They found that hypoxia inhibited the
differentiation of Rcho-1 trophoblasts. At 3% O2, there was very little decrease in Id-2
expression, no induction of palladin, and no significant change in cell size or
cytoskeleton arrangement, despite a seven day treatment under differentiating conditions
as described in Materials and Methods. Cells differentiated for seven days in 5% and 8%
O2 showed increasing levels of palladin induction, decreasing levels of Id-2 expression,
and increasing cytoskeletal rearrangement, though levels were still below those seen in
cells differentiated at 21% O2 [6].

These findings led to the hypothesis that HIF was involved in the regulation of
trophoblast differentiation. Indeed, elevated levels of HIF-1α protein were found in cells
cultured in differentiating conditions at 3% O2 as compared to 21% O2 control samples
and the level of HIF-1α protein induction steadily decreased as oxygen percentage
increased. Additionally, studies done using a dual-luciferase assay with the PGK-1-HRE
experimental luciferase reporter, a luciferase reporter gene under the control of the HIF-1
specific-hypoxia response element from phosphoglycerate kinase-1, or the EPO-HRE experimental luciferase reporter, a luciferase reporter gene under the control of hypoxia response element from the erythropoietin gene, which has been shown to be preferentially up-regulated by HIF-2, showed a significant induction of experimental reporters when transfected cells were cultured at 3% oxygen [28-29]. The level of induction dropped at higher percentages of oxygen, in correlation with the level of HIF-1α protein observed.

E. Present Study

1. HIF-2α.

The initial objective of this study was to determine the role of HIF-2α in Rcho-1 trophoblast differentiation. Because placental formation is tightly regulated by HIFs, it is possible that HIF-1α and HIF-2α play complementary roles in controlling trophoblast differentiation and placental formation in vivo. However, very few studies have been done on HIF-2α in trophoblast stem cells. At least one study failed to detect HIF-2α mRNA is TS cells, though mRNA levels increased after differentiation [7]. However, the levels of HIF-2α protein and transcriptional activity were not studied. Similarly, several other studies have reported that HIF-2α is not functional in murine ES cells, possibly due to its specific tissue localization [3, 33]. Preliminary studies by Gultice et al have shown that HIF-2 mRNA is expressed at all levels of oxygen tension (data not shown), though it is unclear if protein levels correlate with this observation. Therefore, the first aim was to examine HIF-2α protein levels in Rcho-1 trophoblasts cultured at various levels of oxygen by Western blot. Further, characteristic transcriptional activity at each oxygen
level was to be determined via the luciferase assay using the EPO-HRE luciferase reporter.

During the course of this investigation it was found that HIF-2α protein is not present at detectable levels in Rcho-1 trophoblasts cultured in hypoxia, even though high levels of HIF-1α were detected. Because the EPO-HRE experimental reporter is not specific to HIF-2α, only preferential, transcriptional activity of HIF-2α could not be determined due to the high levels of HIF-1α observed.

2. **Luciferase constitutive reporters.**

Previous studies also sought to examine the transcriptional activity of HIF-1α in response to hypoxia in the Rcho-1 trophoblast placental stem cell line, using a conventional luciferase dual-reporter assay [6]. Luciferase dual-reporter assays are widely used in a variety of scientific fields to study transcriptional activation [61-64]. In a conventional luciferase dual-reporter assay, cells are transfected with a firefly (*Photinus pyralis*) luciferase gene under the control of a promoter region from a gene of interest and a *Renilla (Renilla reniformis)* luciferase gene under the control of the promoter region that is constitutively expressed. The two different types of luciferase commonly used have evolutionarily distinct chemical reactions, producing different wavelengths of fluorescence, thus allowing them to be quantified sequentially from a single sample (Figure 6). As experiments are typically performed on independently transfected plates, it is necessary to account for differences in transfection efficiency between plates before results can be analyzed. This is generally accomplished by normalizing the amount of luciferase produced by the experimental firefly luciferase reporter to the amount of
Figure 6: Firefly and Renilla luciferase reporters and their biochemical reactions.

A. Experimental luciferase reporters consist of the firefly luciferase gene under the control of the promoter region from a target gene that is affected by treatment. Control luciferase reporters consist of the Renilla luciferase gene under the control of a promoter from a gene that can be constitutively active in mammalian cells but not affected by treatment. Activation of the promoter regions leads to transcription of the luciferase gene and the accumulation of luciferase. B. The firefly and Renilla luciferase genes are evolutionarily distinct and fluoresce in response to different substrates. This allows both luciferase products to be obtained sequentially from a single sample when using a dual-luciferase assay. Firefly luciferase is quantified first using an oxidizing substrate. This reaction is quenched and the Renilla luciferase is quantified with Stop and Glo reagent [70].
A

**Experimental reporter**

| Target promoter | Firefly lux |

**Control reporter**

| Constitutively active promoter | Renilla lux |

B

**Firefly luciferase:** Beetle luciferin $\xrightarrow{\text{LAR (Mg}^2+)\text{ Oxidation}}$ Oxyluciferin

**Renilla luciferase:** Coelenterazine $\xrightarrow{\text{Stop and Glo reagent Catalysis}}$ Coeleteramide
luciferase produced by the constitutive Renilla luciferase reporter in each treatment [65-66].

Three commercially available luciferase reporters are commonly used as normalizing reporters due to their constitutive activity within most cell types. The pRL-SV40 luciferase reporter (GenBank accession number AF025845) is composed of the Renilla luciferase gene under the control of the constitutively active simian-virus 40 (SV40) early enhancer/promoter. Similarly, the pRL-CMV and pRL-TK (GenBank accession numbers AF025843 and AF025846, respectively) are composed of the Renilla luciferase gene under the control of the constitutively active immediate/early promoter/enhancer of cytomegalovirus (CMV) and herpes simplex virus thymidine kinase (HSV-TK), respectively [65-70]. Several other genes that are commonly used as control reporters for transfection studies are also under the control of a constitutively active CMV or SV40 promoters, including the enhanced green fluorescent protein (EGFP)-N1 plasmid and the lacZ plasmid [67-68, 71].

However, during the course of this study, it was consistently observed that the level of activation of the constitutive reporter was significantly higher in hypoxic samples than in normoxic controls. The activation of constitutive reporters has been previously observed in response to steroid treatment, stimulation with lipopolysaccharide, GATA transcription factors, and stress-activated MAP protein kinases [65-69, 71-73]. Therefore, the effect of hypoxia on constitutive luciferase reporters was also examined. Using a more reliable split transfection protocol, this study determined that the hypoxic induction of luciferase constitutive reporters was not species-specific or cell-type dependent. Further, examination of the promoter regions of the luciferase constitutive
reporters determined that no HREs were present, suggesting that HIF-1 was not directly involved in the hypoxic induction of the luciferase constitutive reporters. Further studies are needed to clarify the exact mechanism of hypoxic induction but this study has demonstrated the importance of using the more reliable split-transfection assay regardless of treatment conditions.
MATERIALS AND METHODS

A. Materials

RPMI 1640 with L-glutamine (RPMI), Dulbecco’s Modified Eagle Medium (DMEM), 1x Dulbecco’s Phosphate Buffered Saline (PBS), and 2-mercaptoethanol were purchased from Fisher Scientific. Fetal Bovine Serum (FBS) was purchased from BioWest. HEPES buffer, Trypsin-EDTA, and antibiotic-antimycotic were purchased from Invitrogen. Sodium pyruvate solution was purchased from VWR. NXTRACT CelLytic™ NuCLEAR™ Extraction Kit was purchased from Sigma-Aldrich. Rabbit polyclonal HIF-1α, rabbit polyclonal HIF-2α and mouse monoclonal HIF-2α antibody (NB100-449, NB100-122, NB100-132) were purchased from Novus Biologicals. Anti-rabbit horseradish peroxidase-conjugated secondary antibody was purchased from BD Transduction. Supersignal West Pico Chemiluminescent substrate was purchased from Pierce. Desferroxamine (DFO) and cobalt chloride (CoCl₂) were purchased from Sigma. The Dual-Luciferase Assay (DLR) kit, pRL-CMV luciferase constitutive reporter plasmid, pRL-SV40 luciferase constitutive reporter plasmid, and pRL-TK luciferase constitutive reporter plasmid were purchased from Promega. Metafectene transfection reagent was purchased from Biontex (Martinsried/Planegg, Germany). The EPO-Hypoxia Response Element (HRE)-luciferase reporter plasmid, with four copies of the HRE consensus sequence from the promoter of the erythropoietin gene in the pGL3 vector was a kind gift of Dr. Florent Soubrier, INSERM, Paris, France. The PGK-1-HRE luciferase reporter plasmid, which contains six copies of a 24 base pair sequence
including the HRE sequence from the phosphoglycerate kinase-1 (PGK-1) promoter upstream of the TK luciferase promoter in the pGL3 vector, was a kind gift of Dr. Peter Ratcliffe, University of Oxford, England. The pc3DNA HIF-1α 3xSDM construct was a kind gift of Dr. Christine Warnecke, University Erlangen-Nuremberg, Erlangen, Germany. The Rcho-1 trophoblast cell line was a kind gift of Dr. Michael Soares, Kansas University Medical Center, Kansas City, Kansas. The mouse monoclonal pan-actin antibody was a kind gift of Dr. James Lessard, Cincinnati Children’s Research Foundation, Cincinnati, Ohio. The Cos 7 and NIH-3T3 cell lines were obtained from ATCC.

B. Cell culture:

1. Rcho-1 Trophoblasts

i. Normal Stem Cell Culture

The Rcho-1 placental trophoblast cell line was cultured as previously described [6, 22, 26]. Briefly, proliferative stem cells were passaged at subconfluence in CM20 media (RPMI, 20% FBS, 50uM 2-mercaptoethanol, 1mM sodium pyruvate solution, 20mM HEPES buffer, 1% antibiotic-antimycotic). Cells were not cultured higher than passage 25, to prevent any accumulated mutations from affecting results. Cell number and viability were determined by Trypan Blue exclusion.

ii. Differentiation

Rcho-1 trophoblasts were differentiated by serum replacement as previously described [6, 22, 26]. Briefly, Rcho-1 cells were plated and allowed to proliferate for three days in CM20 media at 21% O₂ and 37 C. Differentiation media (NCTC-135
medium (Sigma), 50uM 2-mercaptoethanol, 1mM sodium pyruvate, 10% horse serum, and antibiotic) was applied to cells on the third day (day 0) following plating. Media was changed on days 1, 3, and 5, with collection, fixing, or analysis of cells on day 7. For cells differentiated in hypoxia, media was allowed to equilibrate inside the hypoxia chamber at least thirty minutes prior to application to minimize oxygen variation. Cells maintained in CM20 media run concurrently as a control.

iii. Hypoxic Incubation

For hypoxic incubation, cells were plated and allowed to settle in normoxia (21% O₂) overnight or for 7 days, as noted. Cells were then transferred to a Coy hypoxia chamber (Coy Laboratories, Grass Lake, Michigan) for incubation at the desired oxygen tension. The chamber was maintained at 37 °C. For nuclear extracts, cells in hypoxia were collected from the plate while inside the chamber. All reagents were allowed to equilibrate for thirty minutes prior to use on cells within the hypoxia chamber to minimize oxygen exposure. For luciferase assays, cells in hypoxia were removed from the chamber and immediately processed with lysis buffer.

2. Other cell lines

Cos 7 and NIH-3T3 cells were cultured in DMEM, 10% FBS, and 1% antibiotic-antimycotic.
C. Cell Collection

1. Whole Cell Lysates

Whole cell lysates of Cos 7 cells were collected in Buffer D (20mM Tris pH 7.5, 1% NP40, 10% glycerol, 0.2mM 0.5M EDTA pH 8.0) plus protease inhibitor cocktail, sonicated, and centrifuged 15 minutes at 14,500 rpm. Rcho-1 lysates were collected in Buffer D plus protease inhibitor cocktail and proteasome inhibitor. Lysates were sonicated and centrifuged 15 minutes at 14,500 rpm. Protein concentration was determined using Bradford reagent [74]. Lysates were stored at -80 C until use.

2. Nuclear Extraction

Nuclear extracts were collected using the NXTRACT CelLytic™ NuCLEAR™ Extraction Kit per the manufacturer’s instructions. Briefly, cells were washed in 1x PBS three times and then gently scraped off the plate in 1xPBS with a rubber spatula. Cells were spun down at 450 x g for five minutes at 4 C and the supernatant was removed. Packed cell volume (PCV) was estimated and three times that volume of 1x hypotonic lysis buffer plus DTT and protease inhibitor cocktail was used to resuspend the cells. Resuspension was incubated on ice 15 minutes. 10% IGEPAL CA-360 detergent (non-ionic NP-40) was added to a final concentration of 6% and cells vortexed vigorously for 10 seconds to lyse outer membrane. Cells were centrifuged immediately for 30 seconds at 4° C and the cytoplasmic supernatant was removed to a fresh tube. The remaining pellet was resuspended in 2/3 the PCV Extraction Buffer plus DTT and protease inhibitor cocktail. The resuspension was vortexed on high speed for 30 minutes at 4° C then centrifuged 5 minutes. Nuclear supernatant was transferred to clean, chilled tube.
Nuclear and cytoplasmic fraction protein concentrations were determined using Bradford reagent and stored at -80 C until use [74].

**D. Western Blot Analysis**

50 µg of protein was heated with Reducing Sample Buffer (15.62 mM Tris, 0.5% SDS, 3.125 % glycerol, 0.625% β-mercaptoethanol, and 0.025% Bromophenol Blue) at 95 °C for 10 minutes, then loaded on an 8% polyacrylamide gel and separated by SDS-PAGE electrophoresis. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane in transfer buffer (25 mM Tris, 192 mM Glycine, and 20% methanol) overnight at 100 mAmp constant voltage; the membrane was stained with Ponceau Red stain to confirm transfer and incubated with blocking buffer (60 mM Tris base, 204 mM NaCl, 5% non-fat milk, and 0.05% Tween-20, pH 7.4) for 2 hours at room temperature [6, 22]. The blot was probed for either HIF-1α, with a rabbit polyclonal primary antibody, or HIF-2α, with a mouse monoclonal or rabbit polyclonal antibody (1:1,000; overnight at 4 °C) and then an HRP-conjugated anti-rabbit or anti- mouse secondary (1:50,000; 45 minutes at room temperature). Where indicated, the blot was also probed for actin to confirm equal loading using a mouse monoclonal antibody against pan-actin (1:4,000; overnight at 4 °C) and an HRP-conjugated anti-mouse secondary as described. Proteins were visualized using chemiluminescence and Kodak X-ray film (XAR-5).

The blots were stripped of antibody for reprobing using stripping buffer (25mM glycine-HCL and 1% SDS, pH 2.0). The membrane was covered with stripping buffer and incubated at 56° C for 45 minutes. The blot was rinsed and reblocked in blocking buffer for 60 minutes, then incubated with the appropriate secondary antibody (1:50,000).
for 60 minutes at room temperature. Chemiluminescence was applied and the stripped blot was exposed to film to ensure that all antibodies had been removed. After a thorough rinsing in 1xPBS-T, the blot was reincubated with primary antibody as described above.

Densitometric analysis of Western blots was performed using ImageJ software as previously described [75-76].

E. Conventional Luciferase Assay

Conventional luciferase assays were performed as illustrated (Figure 7A). Rcho-1 cells (2x10⁵ cells/ml) were transfected with 5 μl Metafectene, 1 μg EPO-HRE reporter or 1 μg PGK-1-HRE plasmid and 0.2 μg constitutive reporter plasmid, for 18 hours. The DNA plasmid and Metafectene were allowed to complex at room temperature for 20 minutes in serum-free, antibiotic-free media before application to cells [77].

Twenty-four hours post-transfection, one set of samples was moved to a Coy hypoxia chamber (Coy Laboratories, Grass Lake, Michigan) set at 3%, 5%, or 8% O₂ with 5% CO₂/ N₂ balance while the parallel set was maintained at 21% O₂/ 5% CO₂ (normoxia). Cells were collected, analyzed and luciferase reporter activity was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions and measured using a Dynex Revelation 4.06 luminometer (Dynex Technologies, Chantilly, Virginia) [77]. For analysis, the experimental reporter was normalized to the constitutive reporter to control for differences in transfection efficiency. Luciferase activity from hypoxic samples was then compared to the luciferase
Figure 7: Luciferase reporter assays using conventional transfection protocol and equal density passaging of single transfections.

A) In the conventional luciferase assay, two or more plates are plated with equal cell number and individually transfected with identical experimental and constitutive luciferase reporter constructs. During analysis, differences in initial transfection efficiency are accounted for by normalizing the luminescence obtained for the experimental reporter to the luminescence obtained for the constitutive reporter. B) In the split transfection assay, one plate of cells is transfected. That plate is then split equally to two or more plates. However, the luminescence obtained for the experimental reporter can be analyzed without normalizing as equal transfection efficiency between plates is ensured by splitting the original transfection equally prior to treatment.
**A**

Co-Transfection of Experimental (Firefly) and Constitutive (Renilla) Luciferase Reporters

- Plate 1: Experimental Treatment
  - Lysate collected. Luminescence for both reporters recorded with luminometer.
  - Firefly luciferase transfection efficiency normalized to Renilla luciferase
- Plate 2: Control
  - Lysate collected. Luminescence for both reporters recorded with luminometer.
  - Firefly luciferase transfection efficiency normalized to Renilla luciferase

Compare experimental to control results

**Conventional Dual Luciferase Reporter Assay**

**B**

Transfection of Desired Luciferase Reporters

- Plate 1: Experimental Treatment
  - Lysate collected. Luminescence for both reporters recorded with luminometer.
  - Lysate collected. Luminescence for both reporters recorded with luminometer.
- Plate 1b: Control
  - Lysate collected. Luminescence for both reporters recorded with luminometer.

Split equally; transfection efficiency equal

Compare experimental to control results

**“Split Transfection” Luciferase Reporter Assay**
activity in the normoxic sample to determine a fold-change in reporter induction in hypoxia.

**F. Split Transfection Luciferase Assay**

Split transfections were performed as illustrated (Figure 7B). For hypoxic experiments, one plate of Rcho-1, Cos 7, or NIH-3T3 cells (1x10^5 cells/ml) was transfected with 5 µl Metafectene, 1 µg EPO-HRE or 1 µg PGK-1-HRE reporter and 0.2 µg of either pRL-CMV luciferase constitutive reporter, pRL-SV40 luciferase constitutive reporter, or pRL-TK luciferase constitutive reporter. DNA and Metafectene were mixed and incubated at room temperature for 20 minutes in serum-free, antibiotic-free media then applied to cells for 18 hours at 37 C/ 5% CO2.

Twenty-four hours post-transfection, the cells were trypsinized and split equally, as determined by cell number, into two sets of 60mm tissue culture dishes. 24 hours later, one set of plates from each constitutive reporter was moved to the Coy hypoxia chamber, set at 5% O2, for 18 hours while the other set was maintained at 21% O2. The cells were then washed in cold 1xPBS and incubated in 500ul 1xPassive Lysis Buffer for 15 minutes. The cells were removed from the plate with a rubber spatula and centrifuged for 7 minutes at 11,000 rpm to remove debris. Supernatants were used for further analysis. Luciferase reporter activity was determined using the Dual-Luciferase Reporter Assay System and measured on a Dynex Revelation 4.06 luminometer (Dynex Technologies, Chantilly, Virginia). For split-transfections, where transfection efficiency is the same in all samples, the induction of the reporter of interest was directly compared to the induction of the same reporter in the normoxic control.
To examine the effect of artificially-induced HIF stabilization, one plate of Rcho-1 cells (1x10^5 cells/ml) per constitutive reporter was transfected as above. 24 hours post-transfection, the cells were trypsinized and split equally by volume into three sets of 60mm tissue culture dishes. Cells transfected with each constitutive reporter were treated at 21% O₂ with either 100 µM DFO, 100 µM CoCl₂, or vehicle for 18 hours to mimic hypoxic induction. The cells were processed and analysed for luciferase activity as described earlier.

**G. Sequence Analysis**

Sequence analysis was performed using PATCH promoter analysis Public Version 1.0 with boundary set at 87.5 [78].

**H. Statistics**

Standard deviation and statistical significance were calculated using a two-tailed Student T-test. Significance was set at p < 0.05. All experiments were independently repeated a minimum of three times.
RESULTS

A. HIF-2α

1. HIF-2α protein is not detectable in Rcho-1 trophoblast cells.

Preliminary RT-PCR studies have indicated that HIF-2α mRNA was present in Rcho-1 trophoblasts maintained in the stem state at all levels of oxygen tension (data not shown). However, to date, expression of HIF-2α protein in Rcho-1 trophoblasts has not been reported. To determine if HIF-2α protein expression correlates with the mRNA production, Rcho-1 trophoblasts were plated at 3.1 x 10^5 cells per plate. On day 0, two plates were transferred to the hypoxia chamber set at 3%, 5%, or 8% O₂. Two plates were maintained at 21% O₂. One plate in hypoxia and one plate at normoxia were differentiated, beginning on day 0 and continuing through collection, as described in Materials and Methods. Nuclear extracts were collected on day 7 and 50 µg protein was Western blotted and transferred to PVDF membrane as described in Materials and Methods. Fifty micrograms of whole cell lysate from Cos 7 cells that had been transfected with stable constructs of HIF-1α and HIF-2α, which both have three site directed amino acid mutations, were also run as controls. The blot was incubated with a mouse monoclonal primary antibody to HIF-2α and the HRP-conjugated anti-mouse secondary antibody. Proteins were visualized and captured on film. All experiments were performed independently in triplicate to confirm results.

Protein appears on the blot in Rcho-1 trophoblast nuclear extract lanes at about 150 kDa, at 66 kDa, and at 35 kDa, with a doublet appearing at about 60 kDa, in all lanes,
regardless of oxygen tension at the time of culture. The protein at 35 kDa was used as a fortuitous internal loading control, as it also appeared in the Cos 7 control lanes. Also in the Cos 7 lanes, protein was observed at 66 kDa in both HIF-1α SDM and HIF-2α 3xSDM transfected lanes. Protein was also observed at 120 kDa in the Cos 7 cells transfected with HIF-2α 3xSDM that was absent in their HIF-1α 3xSDM transfected counterparts (Figure 8).

In an attempt to elucidate the cause of the higher molecular weight of HIF-2α seen in Rcho-1 cells, Rcho-1 stem cells were either mock-transfected or transfected three times with wild type, flag-tagged HIF-2α or pc3HIF-2α 3xSDM constructs in order to determine if the higher molecular weight observed in Rcho-1 cells was due to unique modifications of the HIF-2 protein in those cells. Rcho-1 stem cells were also treated with 100uM CoCl₂, a hypoxia mimetic, for 18 hours. Nuclear extracts were collected and proteins were separated by SDS-PAGE as described. Whole cell lysates from Cos 7 cells transfected with the HIF-2α overexpression construct were again used as a control. The blot was probed for HIF-2α using the same mouse monoclonal antibody previously used. Protein again appeared in all Rcho-1 lanes at 150 kDa that was not present in Cos 7 control lanes. Additional protein appeared at 120 kDa in lanes from Rcho-1 stem cells transfected with the HIF-2α plasmids and in the Cos 7 control lane (Figure 9). Though protein did appear at 120 kDa in Rcho-1 lanes, it did not resolve the issue of the protein observed at the higher molecular weight.

In a final attempt to clarify the HIF-2α protein reactivity observed at 150 kDa, Rcho-1 cells were maintained as stem or differentiated for seven days at the indicated oxygen level. Nuclear extracts were collected and 50 µg of protein was Western blotted
**Figure 8: HIF-2α protein levels in stem and differentiated Rcho-1 trophoblasts.**

A) Rcho-1 trophoblasts were cultured in the stem-cell state or differentiated at the indicated oxygen level for 7 days as described in Materials and Methods. Nuclear extracts were collected as described. Cos 7 cells were transfected with either pc3HIF-1α 3xSDM or pc3HIF-2α 3xSDM and whole cell lysates were collected. Proteins were separated by SDS-PAGE then transferred to PVDF membrane. The blot was probed with a mouse monoclonal antibody against HIF-2α (NB100-132) as described in Materials and Methods.  

B) Densitometric analysis of HIF-2α protein levels relative the amount of the internal loading control. Values are normalized to 21% O2, represented as 1.
**A**

![Image showing Western Blot analysis of HIF-2α protein expression in Cos7 and Reho-1 nuclear extracts under varying oxygen conditions.](image)

**B**

Densitometric Analysis of HIF-2α Protein Relative to Internal Loading Control

![Bar graph showing fold change in HIF-2α protein level across different oxygen conditions for Cos7 and Reho-1 stem and differentiated cells.](image)
Figure 9: HIF-2α is not post-transcriptionally modified in Rcho-1 cells.

Rcho-1 trophoblasts were plated and either mock transfected or transfected with a wild-type flag-tagged HIF-2α construct or pc3HIF-2α 3xSDM construct as described in Materials and Methods. Rcho-1 trophoblasts were also plated and treated with 100 µM CoCl₂ for 18 hours. Nuclear extracts were collected as described. Cos 7 cells were transfected with pc3HIF-2α 3xSDM and whole cell lysates were collected. Proteins were separated by SDS-PAGE then transferred to PVDF membrane. The blot was probed with a mouse monoclonal antibody against HIF-2α (NB100-132) as described in Materials and Methods.
and transferred as described. Whole cell lysates of Cos 7 cells overexpressing the pc3HIF-1α 3xSDM or pc3HIF-2α 3xSDM plasmid were run as positive controls. Nuclear extracts of Rcho-1 trophoblasts transfected with pc3HIF-1α 3xSDM or pc3HIF-2α 3xSDM or treated with 100 µM CoCl₂ were also run as controls.

Because the availability of good HIF-2α antibodies is limited, and the antibodies that are available vary in reliability, the blot was probed for HIF-2α using a rabbit polyclonal antibody that reportedly yielded better results than the mouse monoclonal antibody used previously. No protein appeared in any lane at 150 kDa, as seen previously. Protein also did not appear at 120 kDa in any lane with Rcho-1s cultured at any percent of oxygen, or in the lane with CoCl₂ treated Rcho-1 stem cells. However, HIF-2α protein was observed at the expected 120 kDa in both lanes where cells had been transfected with the HIF-2α over-expression construct. Additionally, no cross reactive bands were present at lower molecular weights in any lane, in contrast to earlier results. The blot was also incubated with a mouse monoclonal pan-actin antibody to confirm equal loading (Figure 10, top and bottom).

Due to the absence of HIF-2α protein in any lane with treated samples, the results needed to be confirmed. To do this, the blot was stripped as described in Materials and Methods and reprobed for HIF-1α using a polyclonal antibody that had been shown to be reliable. Protein appeared at 120 kDa in all treatment lanes, with higher intensity bands at lower levels of oxygen and in CoCl₂ treated cells. Additionally, protein appeared at 120 kDa in lanes with Cos 7 and Rcho-1 cells transfected with HIF-1α over-expression, but not HIF-2α over-expression, constructs (Figure 10, middle).
Figure 10: HIF-1α but not HIF-2α protein is present in Rcho-1 trophoblasts

A) Rcho-1 trophoblasts were cultured in the stem-cell state or differentiated at the indicated oxygen level for 7 days as described in Materials and Methods. Rcho-1 trophoblast stem cells were also transfected three times with either pc3HIF-1α 3xSDM or pc3HIF-2α 3xSDM as described. Nuclear extracts were collected as described. Cos 7 cells were transfected with either pc3HIF-1α 3xSDM or pc3HIF-2α 3xSDM and whole cell lysates were collected. Proteins were separated by SDS-PAGE then transferred to PVDF membrane as described. Membrane was probed with a rabbit polyclonal antibody against HIF-2α and then with a mouse monoclonal antibody against pan-actin. The blot was then stripped as described and reprobed with a rabbit polyclonal antibody against HIF-1α as described in Materials and Methods. B) Densitometric analysis of HIF-2α and HIF-1α protein levels relative the amount of the actin. Values are normalized to the appropriate 21% O₂ control, represented as 1.
A

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<th>Cos 7</th>
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<td>+ H2 3xSDM</td>
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B

Densitometric Analysis of HIF-2α and HIF-1α Protein Relative to Actin Loading Control
2. **HIF-2α transcriptional activity in Rcho-1 trophoblasts cannot be determined using the EPO-HRE luciferase reporter.**

The luciferase dual-reporter assay was used to determine the transcriptional activity of HIF-2α in Rcho-1 trophoblasts in an attempt to confirm the results of the western blots. The EPO-HRE luciferase experimental reporter was used as it has been shown to be preferential for HIF-2 binding [28-29]. Using the split transfection luciferase assay, Rcho-1 cells were plated at 1.0 X 10⁵ cells and transfected as described in Materials and Methods with 1 µg of EPO-HRE luciferase experimental (firefly) reporter plasmid and 0.2 µg of pRL-SV40 luciferase constitutive (Renilla) reporter plasmid. The transfection was applied overnight, after which cells were given a twenty-four hour recovery period. The plate of transfected cells was then split equally by cell number to two plates. Twenty-four hours later, one plate was moved to the indicated level of hypoxia for 18 hours while the other plate was maintained at 21% O₂ as a control. Cells were lysed, collected, and analyzed as described in Materials and Methods. The results were normalized using the traditional luciferase assay method to ensure that transfection efficiency was equal between samples. In this method, the data for the experimental reporter is normalized to the data for the constitutive reporter before it is compared to other samples. There was no significant induction of the EPO-HRE luciferase reporter at any level of hypoxia in Rcho-1 trophoblasts when the conventional method of normalizing transfection efficiency was used (Figure 11A). The induction of the HIF-1 specific luciferase reporter, PGK-1-HRE, was also examined using the protocol described above. Surprisingly, there was no significant induction of the PGK-1-HRE luciferase reporter when the conventional luciferase assay was used, even though
Figure 11: Fold induction of EPO-HRE and PGK-1 HRE luciferase reporters using the conventional luciferase assay normalizing method.

Rcho-1s were plated at a density of $1.0 \times 10^5$ cells and incubated 24 hours, then transfected with EPO-HRE and pRL-SV40 luciferase reporters as described in Materials and Methods. Samples were treated at the $O_2$ concentration indicated and analyzed as described in Materials and Methods. Results are average of three independent experiments, normalized to values obtained for 21% $O_2$, represented as 1. Error bars represent standard deviation, * $p \leq 0.05$. A) Induction of EPO-HRE luciferase reporter was normalized to induction of pRL-SV40 luciferase constitutive reporter, per conventional luciferase assay protocol. B) Induction of PGK-1-HRE luciferase reporter was normalized to induction of pRL-SV40 luciferase constitutive reporter, per conventional luciferase assay protocol.
A. Induction of EPO-HRE Luciferase Reporter, Normalized to pRL-SV40 Luciferase Constitutive Reporter, by Hypoxia

B. Induction of PGK-1-HRE Luciferase Reporter, Normalized to the pRL-SV40 Luciferase Constitutive Reporter, by Hypoxia
PGK-1 is a direct HIF-1 target (Figure 11B) [29, 31, 33].

However, because the split transfection assay was used, it was possible to directly compare the induction of the experimental reporters at various levels of hypoxia, without first normalizing them to the luciferase constitutive reporters. The results were examined using this method to see if they were comparable to the conventional method of normalizing. Surprisingly, the EPO-HRE luciferase reporter was significantly induced at all levels of hypoxia in Rcho-1 trophoblasts, despite the absence of HIF-2α protein by Western Blot and contradicting the results obtained after normalizing with the constitutive reporter (Figure 12A). As expected, the PGK-1-HRE luciferase reporter was also significantly induced when split transfection analysis was used (Figure 12B)

**B. Luciferase Constitutive Reporters**

1. *Luciferase constitutive reporters are induced by hypoxia.*

Other studies in our laboratory had also examined the transcriptional activity of HIF-1α in Rcho-1 trophoblast stem cells utilizing the conventional luciferase dual-reporter assay. To determine the effects of hypoxia on HIF-1 activity, Rcho-1 trophoblast cells were transfected with either the PGK-1-HRE or EPO-HRE experimental luciferase reporter plasmid, which contains the firefly luciferase gene under the control of six copies of the consensus HRE sequence from PGK-1 or four copies of the consensus HRE sequence from EPO, respectively, and the pRL-SV40 constitutive luciferase reporter. Both PGK-1 and EPO have been shown to be direct targets of HIF-1 [5-6, 29, 36, 43, 50, 57-60]. The transfected cells were then exposed to varying levels of hypoxia
Figure 12: Fold induction of EPO-HRE and PGK-1 HRE luciferase reporters using the split transfection luciferase assay normalizing method.

Rcho-1s were plated at a density of 1.0 x 10^5 cells and incubated 24 hours, then transfected with EPO-HRE and pRL-SV40 luciferase reporters as described in Materials and Methods. Samples were treated at the O₂ concentration indicated and analyzed as described in Materials and Methods. Results are average of three independent experiments, normalized to values obtained for 21% O₂, represented as 1. Error bars represent standard deviation, * p ≤ 0.05. A) Induction of EPO-HRE luciferase reporter by hypoxia, using the split transfection assay protocol. B) Induction of PGK-1-HRE luciferase reporter by hypoxia, using the split transfection assay protocol.
A  Induction of EPO-HRE Luciferase Reporter in Rcho-1 Trophoblast Stem Cells

B  Induction of PGK-1-HRE Luciferase Reporter in Rcho-1 Trophoblast Stem Cells
(3-8% O₂) or normoxia (21% O₂) and the amount of luciferase was quantified by conventional luciferase assay to determine changes in HIF-1 activity.

We observed that the level of fluorescence recorded for the pRL-SV40 constitutive reporter alone was significantly higher in hypoxic samples compared to the constitutive reporter in normoxic controls (Figure 13). Maximal induction occurred with exposure to 5% O₂, with a 9-fold induction of the pRL-SV40 constitutive reporter. A 5-fold induction of the pRL-SV40 constitutive reporter was observed at 8% O₂ and, a 3-fold induction of the reporter was seen at 3% O₂, indicating that the level of pRL-SV40 constitutive reporter induction in hypoxia was highly variable. Because the pRL-SV40 constitutive reporter is used to normalize for transfection efficiency between samples, high levels of variability introduce an increase in potential errors within our data analysis.

In order to minimize potential variability in our data collection, we instituted an alternate transfection protocol that normalizes transfection efficiency between samples prior to treatment. This method allows experimental (firefly) reporter induction to be directly compared between samples without needing a constitutive (Renilla) reporter to normalize for transfection efficiency (Figure 7B).

In light of the high degree of variability, the pRL-SV40 luciferase constitutive reporter data that had been used to normalize the EPO-HRE luciferase data in the HIF-2α study was examined. Because the split transfection assay was used, the amount of constitutive reporter activity should be equal. However, in comparison to constitutive reporter activity at 21% O₂, set as 1, the pRL-SV40 luciferase constitutive reporter was significantly induced by all levels of hypoxia (Figure 14).
Figure 13: Fold increase in pRL-SV40 luciferase constitutive reporter activity in Rcho-1 trophoblast cells in hypoxia.

Parallel plates of Rcho-1 trophoblast cells were plated at equal cell number of 1.0 x 10^5 cells and transfected with 1 µg EPO-HRE and 0.2 µg pRL-SV40, using the conventional luciferase assay. Samples were treated at the O_2 concentration indicated and analyzed as described in Materials and Methods. Results are the average of three independent experiments, normalized to values obtained for 21% O_2, represented as 1. Error bars represent standard deviation, * p ≤ 0.05.
Variability of pRL-SV40 Induction in Hypoxia in Rcho-1 Trophoblasts in Conventional Dual Luciferase Assays

Fold Change in Luciferase Activity

Percent Oxygen

3%  5%  8%  21%

*
Figure 14: Fold induction of pRL-SV40 luciferase constitutive reporter after exposure to hypoxia, using the split transfection method.

The split transfection method was used. Rcho-1s were plated at a density of 1.0 x 10^5 cells and incubated 24 hours, then transfected with EPO-HRE and pRL-SV40 luciferase reporters as described in Materials and Methods. Samples were treated at the O_2 concentration indicated and analyzed as described in Materials and Methods. Induction of pRL-SV40 luciferase constitutive reporter alone, after exposure to hypoxia is shown. Results are an average of three independent experiments, normalized to values obtained for 21% O_2, represented as 1. Error bars represent standard deviation, * p \leq 0.05.
Hypoxic Induction of pRL-SV40 Luciferase Constitutive Reporter in Rcho-1 Trophoblast Stem Cells

Induction of pRL-SV40 Luciferase Reporter in Rcho-1 Trophoblast Stem Cells

Fold Change in Luciferase Activity

Percent Oxygen

3% 5% 8% 21%

*
To determine if this hypoxic induction was limited to pRL-SV40, we repeated the split transfection assay with two other commonly used commercially available constitutive luciferase reporters, pRL-CMV and pRL-TK. Our results indicate that pRL-CMV is also significantly induced by hypoxia (~ 3-fold). Plasmid RL-TK induction by hypoxia did not reach statistical significance (Figure 15A). Similar, but not identical, results were obtained when the PGK-HRE experimental reporter was used, with significant hypoxic induction of both pRL-CMV and pRL-TK constitutive luciferase reporters, indicating that the hypoxic induction was most likely independent of the experimental reporter (Figure 15B).

2. Hypoxic induction of luciferase constitutive reporters is not species or cell-type specific.

Rcho-1 trophoblasts are a rodent, choriocarcinoma-derived placental stem cell line that serves as a model of the giant cell placental lineage [6, 22, 26]. To determine if the induction of constitutive luciferase reporters by hypoxia was restricted to the placental cell type, the split transfection assays were repeated in Cos-7 cells, an immortalized, SV40-transformed African green monkey kidney cell line [79], and NIH-3T3 cells, a primary mouse embryonic fibroblast cell [80]. The EPO-HRE experimental luciferase reporter was co-transfected with the pRL-SV40, pRL-CMV, or pRL-TK constitutive luciferase reporters. In Cos 7 cells, both pRL-SV40 and pRL-CMV were significantly induced (1.5-fold and 3.25-fold, respectively) by exposure to hypoxia of 5% O₂ (Figure 16). As in Rcho-1 trophoblasts, pRL-TK was induced by exposure to
Figure 15: Induction of luciferase constitutive reporters by hypoxia in Rcho-1 trophoblasts with EPO-HRE and PGK-1-HRE reporters.

The split transfection method was used. Equal numbers of Rcho-1 cells were plated and incubated 24 hours. Cells were transfected with A) 1 µg EPO-HRE luciferase reporter and 0.2 µg pRL-SV40, pRL-CMV, or pRL-TK constitutive luciferase reporter or B) 1 µg PGK-1-HRE luciferase reporter and 0.2 µg pRL-SV40, pRL-CMV, or pRL-TK constitutive luciferase reporter as described in Materials and Methods. Samples were treated at the O₂ concentration indicated and analyzed as described in Materials and Methods. Results are average of three independent experiments, normalized to values obtained for 21% O₂, represented as 1. Error bars represent standard deviation, * p ≤ 0.05.
A

Hypoxic Induction of Constitutive Luciferase Reporters in Rcho-1 Trophoblast Stem Cells Co-Transfected with EPO-HRE Luciferase Reporter

![Graph A](image)

B

Hypoxic Induction of Constitutive Luciferase Reporters in Rcho-1 Trophoblast Stem Cells Co-Transfected with PGK-1-HRE Luciferase Reporter

![Graph B](image)
Figure 16: Induction of luciferase constitutive reporters by hypoxia in Cos 7 cells.

The split transfection method was used. Twenty-four hours after plating, Cos 7 cells were transfected with 1 µg EPO-HRE luciferase reporter and 0.2 µg pRL-CMV, pRL-SV40, or pRL-TK constitutive luciferase reporter as described in Materials and Methods. Samples were treated at the O$_2$ concentration indicated and analyzed as described in Materials and Methods. Results are average of three independent experiments, normalized to values obtained for 21% O$_2$, represented as 1. Error bars represent standard deviation, * p ≤0.05.
Hypoxic Induction of Constitutive Luciferase Reporters in Cos 7 Cells

![Graph showing fold change in luciferase activity under 21% O2 and 5% O2 for SV40, CMV, and TK constitutive reporters.](image-url)
hypoxia, but not to the level of statistical significance. In NIH-3T3 cells, pRL-CMV showed a significant, nearly 3-fold induction upon exposure to hypoxia of 5% O₂, while pRL-SV40 and pRL-TK had only a slight induction that was not statistically significant (Figure 17).

3. Hypoxic mimetics do not induce luciferase constitutive reporters.

To evaluate whether consensus HRE or HRE-like sequences played a role in the hypoxic induction of the constitutive luciferase reporters, we next examined the induction of constitutive luciferase reporters after treatment with compounds, such as DFO and CoCl₂, that act to stabilize HIF-1α in vitro and allowing transcriptional activation of HIF-1 [32, 37, 43, 46, 52, 57-60]. Both compounds act by blocking the action of the prolyl hydroxylases which target HIF-α for degradation by hydroxylating conserved proline residues. DFO, an iron chelator, scavenges Fe(II), depleting it as a cofactor. CoCl₂ acts by allowing substitution of Co(II) for Fe(II) as a cofactor of prolyl hydroxylase, preventing its action [1, 34, 45, 49]. These two compounds were used to evaluate the role of HIF in the induction of the constitutive luciferase reporters in Rcho-1 trophoblasts using the split transfection assay. If the binding of consensus HRE or HRE-like sequences by HIF-1 is responsible for the hypoxic induction of the constitutive luciferase reporters, stabilization of HIF-1α by DFO or CoCl₂ would be predicted to cause an increase in the level of constitutive reporter luciferase compared to vehicle-treated samples. Surprisingly, no significant induction of the constitutive luciferase reporters was observed when luciferase transfected Rcho-1 trophoblast cells were treated with the hypoxia mimetics DFO and CoCl₂ compared to vehicle-treated cells (Figure 18A), despite the induction of PGK-1-HRE, HIF-1α specific, luciferase reporter in cells treated
Figure 17: Induction of luciferase constitutive reporters by hypoxia in NIH-3T3 cells.

The split transfection method was used. Twenty-four hours after plating, NIH-3T3 fibroblasts were transfected with 1 µg EPO-HRE luciferase reporter and 0.2 µg pRL-CMV, pRL-SV40, or pRL-TK constitutive luciferase reporter as described in Materials and Methods. Samples were treated at the O₂ concentration indicated and analyzed as described in Materials and Methods. Results are average of three independent experiments, normalized to values obtained for 21% O₂, represented as 1. Error bars represent standard deviation, * p ≤0.05.
Hypoxic Induction of Constitutive Luciferase Reporters in NIH-3T3 Cells

Fold Change in Luciferase Activity

Constitutive Reporter

SV40  CMV  TK

21% O2  5% O2

*
**Figure 18: Induction of PGK-1-HRE and luciferase constitutive reporters by DFO or CoCl\(_2\).**

The split transfection method was used. Equal numbers of Rcho-1 trophoblasts were seeded and incubated 24 hours. Rcho-1 trophoblasts were then transfected with A) 1 µg EPO-HRE luciferase reporter and 0.2 µg pRL-CMV, pRL-SV40, or pRL-TK constitutive luciferase reporter or B) 1 µg PGK-1-HRE luciferase reporter as described in Materials and Methods. Twenty-four hours post transfection, each plate was split equally to three plates. Twenty-four hours later one plate from each reporter set was treated with either 100µM DFO, 100µM CoCl\(_2\), or vehicle for 18 hours. Samples were lysed, collected, and analyzed as described in Materials and Methods. Results are an average of A) three independent experiments, or B) eight independent experiments, normalized to values obtained for 21% O\(_2\), represented as 1. Error bars represent standard deviation, * p ≤0.05.
A

Induction of Luciferase Constitutive Reporters by DFO and CoCl₂ in Rcho-1 Trophoblast Stem Cells

Fold Change in Luciferase Activity

Vehicle
+ DFO
+ CoCl₂

Constitutive Reporter
SV40
CMV
TK

B

Induction of PGK-1-HRE Luciferase Reporter by DFO and CoCl₂ in Rcho-1 Trophoblast Stem Cells

Fold Change in PGK-1-HRE Luciferase Activity

Vehicle
DFO
CoCl₂

*
with DFO and CoCl₂ (Figure 18B). HIF-1α protein was also shown to be stabilized by DFO and CoCl₂, indicating that the two compounds were functional (Figure 19).

4. Transfection of HIF-1α does not induce luciferase constitutive reporters.

HIF-1α is stabilized under low oxygen conditions and studies have shown that protein levels increase when trophoblasts are exposed to hypoxia [4, 29-30, 43]. The mechanism of stabilization of HIF-1α by hypoxia differs from the stabilization with hypoxia mimetics such as DFO and CoCl₂. Therefore, we examined the induction of luciferase constitutive reporters after transfecting Rcho-1 cells with a constitutively-active, site-directed mutant HIF-1α construct (pc3HIF-1α 3xSDM) that is stable under normoxic conditions [29]. The pc3HIF-1α 3xSDM construct is stably expressed and active in normoxia due to two site directed point mutations within the oxygen dependent degradation domain and one site directed point mutation within the transactivation domain at the sites of hydroxylation responsible for its usually rapid normoxic turnover [29].

Rcho-1 trophoblasts were transfected with the EPO-HRE or PGK-1-HRE luciferase reporter along with one of the three constitutive luciferase reporters and then plated at equal cell number into four parallel samples, using the split transfection method. One plate was mock-transfected while the other three were transfected with either pc3DNA, pEGFP, or pc3HIF-1α 3xSDM. If HIF-1 plays a role in the induction of constitutive luciferase reporters, the induction seen in cells transfected with the stable construct should be higher than in mock-transfected cells, cells transfected with only the vector backbone or cells transfected with EGFP. Results, however, indicate that
Figure 19: Induction of HIF-1α protein by DFO and CoCl2 in luciferase transfected Rcho-1 trophoblasts.

A) The split transfection method was used. Equal numbers of Rcho-1 trophoblasts were seeded and incubated 24 hours. Rcho-1 trophoblasts were then transfected with 1 µg PGK-1-HRE luciferase reporter and 0.2 µg pRL-CMV, pRL-SV40, or pRL-TK constitutive luciferase reporter as described in Materials and Methods. Twenty-four hours post transfection, each plate was split equally to three plates. Twenty-four hours later one from each reporter set was treated with either 100 µM DFO, 100 µM CoCl2, or vehicle for 18 hours. Nuclear extracts were collected and proteins separated by SDS-PAGE before transfer to PVDF membrane as described in Materials and Methods. Blot probed for HIF-1α and pan-actin as described. Blots shown are representative of three independent experiments. B) Densitometric analysis of HIF-1α protein levels relative the amount of the actin loading control. Values are normalized to 21% O2, represented as 1.
Densitometric Analysis of HIF-1α Protein Relative to Actin Loading Control

Constitutive Reporter and Treatment
transfection with the stable HIF-1α 3xSDM construct failed to induce any of the three constitutive luciferase reporters above levels seen in control transfections (Figure 20A), even though the pc3HIF-1α 3xSDM construct did induce an increase in PGK-1-HRE experimental reporter activity above that of mock transfected, vector transfected, or enhanced GFP transfected cells (Figure 20B). Additionally, the presence of HIF-1α protein in cells triple-transfected with the constitutively active HIF-1α construct was detected by Western blot (Figure 20C). These results indicate that the transcriptional activation of constitutive luciferase reporters in response to hypoxia may be independent of HIF-1.

5. Luciferase constitutive reporter promoter sequences do not contain consensus HREs.

The three constitutive luciferase reporters share a common vector backbone (pRL) but have different functional promoter regions from genes that can be constitutively activated in most mammalian cells. Because the induction of the luciferase constitutive reporters after hypoxic exposure varied between reporters and also between cell types after using the split transfection method, we hypothesized that the sequence or sequences responsible for the observed induction of the constitutive reporters were within the promoter sequences and not within the common backbone [70]. The induction of all three constitutive luciferase reporters occurred after exposure to hypoxia; therefore, we analyzed the promoter sequences for consensus HREs using PATCH promoter analysis software [78]. Analysis of the genetic sequences of SV40, CMV, and TK promoters revealed that there were no "consensus" HRE sequences (5’-T/G ACGTGCGG-3’).
Figure 20: Induction of PGK-1-HRE, luciferase constitutive reporters, and HIF-1α protein by pc3DNA HIF-1α 3xSDM

The split transfection method was used. Equal numbers of Rcho-1 trophoblasts were seeded and incubated 24 hours. Rcho-1 trophoblasts were then transfected with A) 1 µg PGK-1-HRE luciferase reporter or B) 1 µg EPO-HRE luciferase reporter and 0.2 µg pRL-CMV, pRL-SV40, or pRL-TK constitutive luciferase reporter as described in Materials and Methods. Twenty-four hours post transfection, each plate was split equally to four plates. Twenty-four hours later one plate from each reporter set were transfected with either 5 µg pc3DNA, 5 µg pEGFP, 5 µg pc3HIF-1α 3xSDM, or mock transfected with reagent alone. Samples were lysed, collected, and analyzed as described in Materials and Methods. Results are an average of A) three independent experiments or B) six independent experiments, normalized to values obtained for 21% O₂, represented as 1. Error bars represent standard deviation, * p ≤ 0.05. C) The split transfection method was used. Equal numbers of Rcho-1 trophoblasts were seeded and incubated 24 hours. Rcho-1 trophoblasts were then transfected with 1 µg PGK-1-HRE luciferase reporter and 0.2 µg pRL-CMV, pRL-SV40, or pRL-TK constitutive luciferase reporter as described in Materials and Methods. Twenty-four hours post transfection, each plate was split equally to four plates. Twenty-four hours later one plate from each reporter set was transfected with either 5 µg pc3DNA, 5 µg pEGFP, 5 µg pc3HIF-1α 3xSDM, or mock transfected with reagent alone. Nuclear extracts were collected and proteins separated by SDS-PAGE before transfer to PVDF membrane as described in Materials and Methods. Blot was probed for HIF-1α and pan-actin as described. Blots shown are representative of three independent experiments.
A

Induction of PGK-1-HRE Luciferase Reporter by pc3HIF-1α 3xSDM in Rcho-1 Trophoblast Stem Cells

B

Induction of Luciferase Constitutive Reporters by pc3HIF-1α 3xSDM in Rcho-1 Trophoblast Stem Cells

C
present in any of the three constitutive luciferase reporters (Table 1). There was one HIF-1 binding site (5’-TACGTG-3’) present in the TK promoter, but it was not a full consensus HRE sequence and no such sites were found in the other promoters. Several other consensus sequences were found, however, in all three reporters, as listed in Table 1.
Table 1: Consensus Sequences in Luciferase Constitutive Reporters

The promoter regions for each luciferase constitutive reporter were analyzed for consensus sequences using Patch Promoter Analysis software. Selected consensus sequences and their prevalence within each promoter are listed. *Steroid hormone binding factors include RXR, T3R, GR, ER-α and –β, PPAR-γ, FXR, and LXR.
<table>
<thead>
<tr>
<th>Binding Factor</th>
<th>Consensus Sequence</th>
<th>SV40</th>
<th>CMV</th>
<th>TK</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1</td>
<td>5'-TACGTG-3'</td>
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<td>0</td>
<td>1</td>
</tr>
<tr>
<td>AhR/ARNT</td>
<td>5'-T/GNGCGTGA/CG/CA-3'</td>
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<td>2</td>
<td>4</td>
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<tr>
<td>NF-kB</td>
<td>5'-GGGACTTTTCC-3'</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>AP-2</td>
<td>5'-CCCAGGCA-3'</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>CREB</td>
<td>5'-TGACGTCA-3'</td>
<td>0</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Steroid Hormones*</td>
<td></td>
<td>8</td>
<td>26</td>
<td>11</td>
</tr>
<tr>
<td>RXR</td>
<td>5'-GGGGTCA-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>5'-AGGTCAnAGGTC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LXR</td>
<td>5'-AGGTCAnAGGTC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3R</td>
<td>5'-TA/GAGGTCA-3'</td>
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<td></td>
</tr>
<tr>
<td>GR</td>
<td>5'-GGTACAnTGTTC-3'</td>
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</tr>
<tr>
<td>ER</td>
<td>5'-GGTCAnACTGG-3'</td>
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</tbody>
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DISCUSSION

A. HIF-2α

1. HIF-2α protein is not detectable in Rcho-1 trophoblasts.

Through these studies, the contribution of HIF-2α in Rcho-1 trophoblasts was explored and the role of HIF-1α in the hypoxic induction of luciferase constitutive reporters was investigated. Though preliminary studies in our lab identified HIF-2α mRNA expression in Rcho-1 trophoblasts at all levels of oxygen, HIF-2α protein was not detected in nuclear extracts at any level of hypoxia or after treatment with CoCl2, despite high levels of HIF-1α expression. Initially, Western blots indicated that HIF-2α protein levels appeared to correlate with mRNA expression. However, the high molecular weight (150 kDa compared to 120 kDa) of HIF-2α in Rcho-1 trophoblasts was inconsistent with published results. Rcho-1 stem cells were transfected with a stable HIF-2α construct or a wild-type HIF-2α construct that was flag-tagged to determine if a phosphorylation event or other post-translational modification was the cause of the disparity. Low levels of HIF-2α were seen at the correct molecular weight of 120 kDa in cells transfected with HIF-2α constructs. The protein observed in the wild-type transfected cells was most likely caused by overwhelming the degradation pathway with excess protein. However, the higher molecular weight band was still present in these transfected cells, so the disparity was not resolved. Finally, a new polyclonal antibody was used. Surprisingly, no band appeared at the higher molecular weight. HIF-2α was present in Cos 7 and Rcho-1 cells transfected with the stable HIF-2α construct, but not in
any other lane. The blot was reprobed for HIF-1α to confirm these results. Strong HIF-1α expression was observed at 3% and 5% oxygen and in lanes transfected with stable HIF-1α constructs or treated with CoCl₂, with lower levels of HIF-1α at 8% and 21% O₂. It is likely, therefore, that the band originally presumed to be HIF-2α was, in fact, a cross-reactive band between the mouse monoclonal antibody and the rodent protein. This is supported by the absence of the band in Cos 7 (monkey) cells and in Rcho-1 cells when a rabbit polyclonal antibody is used.

2. Model systems of placental formation

From this study, it was concluded that HIF-2α is not present in Rcho-1 cells or is present in such low levels that it is undetectable with the available tools. This correlates with other studies that have found that HIF-2α is not expressed in murine embryonic stem cells, or that protein is present but not active [3, 33]. High levels of HIF-2α protein have been found in vascular endothelial cells and from preeclamptic human placentas, cells which may not have blastocyst origins [5, 8, 20, 30, 33, 35]. The difficulty in examining and characterizing the development of the human placenta lies in the limitations of the cell models available for study. Though murine cell models share many characteristics and have a high degree of homology to their human homologs, there are aspects of development that differ substantially [11, 16, 18-19]. The expression of certain hormones and the spatial-temporal timing of cell differentiation between mice and humans are not conserved and so any findings made using mouse cell lines must be extrapolated carefully to the actual human process.

An additional difficulty in the study of trophoblast differentiation lies in the cell lines themselves. Though all three cell lines commonly used in the study of trophoblasts
have been analyzed and confirmed as being models for their specific lineage, each one
has its own limitations. The TS3.5 cell line differentiates into all murine trophoblast
lineages simultaneously making the study of a specific cell lineage difficult [13, 21-23].
Chemicals can be used to “push” the cells toward one lineage but it is unclear if the
inducing factors are similar \textit{in vivo} [21-23]. Therefore, these studies must be analyzed
critically to ensure that results are truly a lineage-specific phenomenon and not a product
of the chemical treatment. The SM10 and Rcho-1 trophoblast cell lines have been
shown to be committed to differentiate toward the labyrinthine and giant cell lineages,
respectively, making them a more reliable way to study the functions of a particular
lineage [21, 24, 26].

Placental development occurs with intimate contact between trophoblast stem
cell types and between mother and fetus. Additionally, cells presumably receive many
other signals from their environment that contribute to the spatial-temporal timing of their
differentiation. Therefore, paradoxically, it is difficult to fully elucidate the development,
differentiation, and functions of a particular cell lineage in the absence of those signals.
Cell lines with tumor origins, such as the Rcho-1 cell line, must also be used carefully, as
their derivation may lend them characteristics atypical of the lineage they model. Though
studies show that Rcho-1 trophoblast cells display all the characteristics of the actual
giant cell lineage, care must still be taken when results are interpreted [6, 22]. The final
limitation of using derived cell lines is the conditions under which they are cultured.
Trophoblast stem cells become lineage committed and exist in a hypoxic environment [1-
3, 4-7, 11-13]. The gradient of oxygen experienced by cells as the maternal decidua is
invaded and blood flow established is probably also accompanied by other signals, such
as location and cell-cell contact, which all serve to regulate differentiate. These *in vivo* conditions have yet to be fully elucidated and are difficult to replicate accurately in a cell culture system. Furthermore, cell lines are often maintained and many studies are conducted in ambient air (21% O₂), which may shift the cell lines away from their typical *in vivo* characteristics. Despite these problems, the cell lines used to characterize trophoblast differentiation are useful tools and have greatly expanded the body of knowledge concerning placental development and have allowed the molecular mechanisms behind many processes to be elucidated.

**B. Luciferase Constitutive Reporters**

1. *Luciferase constitutive reporters are induced by hypoxia independent of luciferase experimental reporters.*

   The transcriptional activity of HIF-2α was also measured using the luciferase dual-reporter assay, which is very sensitive to changes in transcriptional activity and is quantifiable. In the conventional luciferase assay, plates of cells are transfected with luciferase constructs individually prior to treatment. In order to account for the differences in transfection efficiency between plates, a luciferase constitutive reporter is used to normalize for transfection efficiency of each plate. Because previous studies in our lab had revealed a high level of variability in the constitutive reporter readings after exposure to hypoxia, we began using the split transfection assay. In this protocol, one plate of cells is transfected with luciferase reporters and then split to equal cell numbers, equalizing transfection efficiency between plates. The plates can be treated and experimental luciferase reporter induction can be analyzed without normalizing with a constitutive reporter, eliminating a source of potential error.
Using the split transfection method, Rcho-1 cells were transfected with the EPO-HRE luciferase experimental reporter, which has been shown by several groups to be preferentially targeted by HIF-2α. The pRL-SV40 luciferase constitutive reporter was co-transfected as an additional control. The transfected cells were split equally and incubated at various levels of hypoxia. Using the conventional method of normalizing for transfection efficiency, the results indicated that the EPO-HRE reporter was not activated at any level of hypoxia, supporting the results obtained by Western blot. The pRL-SV40 luciferase constitutive reporter data was analyzed for variance as all readings should be extremely similar when the split transfection assay is used. Surprisingly, pRL-SV40 luciferase constitutive reporter was significantly induced at all levels of hypoxia when compared to the 21% O₂ control. Reexamination of the EPO-HRE luciferase reporter induction using the split transfection method allowing direct comparison revealed that it was significantly induced at all levels of hypoxia, contradicting the results of the HIF-2α Western blot. However, it does correlate with the high levels of HIF-1α protein and indicates that the EPO-HRE luciferase reporter may be preferential for HIF-2α but that it can still be targeted by HIF-1α and may not be ideal for use in cells containing both isoforms.

Two other commonly used luciferase constitutive reporters were also tested alongside the pRL-SV40 luciferase constitutive reporter with both the EPO-HRE and PGK-1-HRE luciferase experimental reporters in Rcho-1 trophoblasts at 5% O₂ as it represents moderate hypoxia. The pRL-CMV luciferase constitutive reporter was significantly induced with both experimental reporters while the pRL-TK constitutive reporter was induced with both experimental reporters but significantly so only with the
PGK-1-HRE constitutive reporter. This indicates that the hypoxic induction is not specific to the pRL-SV40 luciferase constitutive reporter but is common to all three commonly used reporters. The similar induction of luciferase constitutive reporters seen with the EPO-HRE and PGK-1-HRE experimental reporters indicates that the hypoxic induction of the constitutive reporters is independent of the experimental reporter used.

2. Hypoxic induction of luciferase constitutive reporters is not cell-type or species-specific.

The Rcho-1 cell line is a rodent choriocarcinoma derived cell line [6, 22, 26]. To determine if the hypoxic induction of the luciferase constitutive reporters was specific to the Rcho-1 cell line, hypoxic induction of all three reporters was tested in two more cell lines from different species and with different derivations. The Cos 7 cell line is an African green monkey kidney cell line which has been immortalized by SV40-transformation [79]. In contrast, the NIH-3T3 cell lines are primary mouse fibroblast cells [80]. Even in these vastly different cell lines, significant induction of the luciferase constitutive reporters was observed. The pRL-SV40 and pRL-CMV reporters were significantly induced in the Cos 7 line. Plasmid RL-TK was induced but not to statistical significance. Only the pRL-CMV reporter was significantly induced in the NIH-3T3 cell line, though both pRL-SV40 and -TK luciferase constitutive reporters do show induction but not to statistical significance. These results indicate that the hypoxic induction of luciferase constitutive reporters is not cell-type dependent or species-specific.
3. **Stable HIF-1α protein fails to induce luciferase constitutive reporters.**

HIFs are major mediators of gene expression under hypoxic conditions, therefore, it was possible that the hypoxic induction observed was the result of HIF-1 interactions with the luciferase constitutive reporters. To test this, two chemical stabilizers of HIF-1α, DFO and CoCl₂ were applied to Rcho-1 trophoblasts transfected with the luciferase constitutive reporters and either the EPO-HRE or PGK-1-HRE experimental reporters. Both mimetics failed to induce significant levels of luciferase constitutive reporter induction, even though HIF-1α protein was stabilized as determined by Western blot and levels of HIF-1α specific PGK-1-HRE luciferase experimental reporter, a HIF-1α target, were induced. Because both DFO and CoCl₂ disrupt the prolyl and asparaginyl hydroxylases responsible for HIF-1α degradation, they have commonly been referred to as *hypoxia* mimetics. However, the failure of DFO and CoCl₂ to induce luciferase constitutive reporters challenges that label and suggests that hypoxia produces a much broader spectrum of cellular changes than just HIF-1 induction.

A similar experiment was performed to confirm the results obtained using chemical stabilizers of HIF-1α. A HIF-1α construct that has been rendered stable in normoxia by amino acid substitution at the sites of prolyl and asparaginyl hydroxylation was transfected into Rcho-1 trophoblasts transfected with the luciferase constitutive reporters and either the EPO-HRE or PGK-1-HRE experimental reporters. Several other constructs were used as controls. Transfection of the stable HIF-1α construct failed to significantly induce luciferase constitutive reporters above mock-transfected, pc3DNA, or pEGFP-N1 transfected controls. The pc3HIF-1α 3xSDM construct did significantly induce the PGK-1-HRE experimental reporter and did increase HIF-1α protein levels in
Reho-1 trophoblasts as seen by Western blot. These results support those obtained using chemical stabilizers of HIF-1α and indicate that a different mechanism is responsible for the induction of luciferase constitutive reporters in hypoxia.

4. Luciferase constitutive reporters contain diverse consensus sequences.

Together, the results obtained using DFO, CoCl₂, and the normoxically stable HIF-1α construct indicate that the hypoxic induction of luciferase constitutive reporters is not directly associated with HIF-1α. All three luciferase constitutive reporters have identical backbones and only differ in their respective promoter regions [70]. Additionally, the amount of hypoxic induction varied between the luciferase constitutive reporters in the same cell type. This suggested that the cause of the induction was a factor whose binding site is present in varying numbers within the promoter regions of all three luciferase constitutive reporters. Further, this factor is probably differentially expressed across cell types, which would account for the variation in hypoxic induction between the luciferase constitutive reporters in different cell types.

Patch promoter analysis software was used to analyze the promoter regions from all three luciferase constitutive reporters for consensus sequences. As expected, no consensus HRE sequences were present in any of the reporters. One partial HIF-1 binding site was found within the pRL-TK reporter, which showed the least amount of significant induction across all three different cell types tested, supporting the supposition that hypoxic induction of the luciferase constitutive reporters was independent of HIF-1. Several consensus ARNT binding sites were found within the pRL-CMV and pRL-TK reporters [81-82]. None were present in the pRL-SV40 promoter, making it unlikely to be responsible for the hypoxic induction of the luciferase constitutive reporters.
Similarly, the consensus cAMP response element binding protein (CREB), which has been shown to be up-regulated under hypoxic conditions, was only present in the pRL-CMV and pRL-TK luciferase constitutive reporters [83-84]. While the high number of CREB sites found in pRL-CMV may contribute to the high induction seen in that reporter, it is unlikely the primary cause of hypoxic induction as it is not found in all three luciferase constitutive reporters.

Three consensus binding sequences were found in all three constitutive reporters: nuclear factor -κB (NF-κB), AP-2, and a Steroid Hormone Response Element (StRE). The StRE sites contained binding sites for a wide range of steroid receptors including retinoic acid receptors (RAR), retinoic x receptors (RXR), and the related heterodimer partners liver x and farnesoid x receptors (LXR and FXR, respectively). Other steroid binding sites represented are peroxisome proliferator-activated receptor-γ (PPAR-γ), thyroid hormone receptor (T3R), estrogen receptor -α and -β (ERα and ERβ), and glucocorticoid receptor (GR). All of these factors have the potential to be up-regulated under a hypoxic stimulus and can function independently of HIF-1 [85-106].

NF-κB is a highly-conserved transcription factor found in all cells and active in many signaling pathways for cell growth and proliferation. It is also responsible for a number of cellular responses to inflammation or stress, particularly oxidative stress, to protect cells from apoptosis [85-90]. There were five consensus NF-κB binding sites in the pRL-SV40 promoter, four in the pRL-CMV promoter, and one in the pRL-TK promoter, which correlate with the relative induction of each reporter in hypoxia. NF-κB up-regulation in response to the generation of reactive oxygen species or stress from
exposure to hypoxia may be responsible for the induction of the luciferase constitutive reporters [88-90].

AP-2 is another transcription factor that mediates gene expression during cellular stress responses [91-93]. It is also required during mammalian development for normal growth and morphogenesis [93-94]. AP-2 can be induced by retinoic acid, cAMP, and singlet oxygen [91]. It is involved in the formation of trophoderm cells and required for kidney formation, supporting its role in regulating cell proliferation. AP-2 also plays in a role in the suppression of terminal differentiation of cells during embryonic development [4, 6, 29, 93]. Three consensus AP-2 binding sites were found in the pRL-SV40 and pRL-TK luciferase constitutive reporters and four were found in the pRL-CMV reporter, indicating that AP-2 may also be involved in the hypoxic induction of luciferase constitutive reporters.

The steroid hormone binding site (StRE) provides a promising explanation for the hypoxic induction of luciferase constitutive reporters. As listed earlier, the array of hormones that can bind to this site is responsible for the regulation of a number of different responses and pathways. The retinoic acid receptor family (RAR, RXR, LXR, FXR) binds retinoids such as vitamin A. Retinoids regulate processes that include cell growth, differentiation, and development. Upon binding, the retinoic acids act as hetero- or homodimeric transcription factors and have been shown to protect cells from hypoxically associated cell death by inhibiting c-Jun and p38, a MAP kinase [95-97]. Several different retinoid response pathways have been identified; the most common retinoid signaling pathways are the RXR/RAR and RXR/RXR pathways [99]. LXR and FXR also heterodimerize with RXR to mediate responses in an organ-specific pattern.
LXR has no known ligands but mediates responses in the visceral organs while the ligands for FXR are natural bile acids. FXR mediates responses in the kidney, liver, adrenal gland, and ilium [98-100]. PPARs are another family of transcription factors closely related to retinoic acid receptor family. PPARs mediate differentiation and cellular metabolism; the most commonly expressed isoform is PPAR-γ. PPAR-γ exists in three isoforms (γ₁-γ₃) with PPAR-γ₁ being most widely expressed. PPARs also dimerize with RXR to mediate the formation of peroxisomes to rid cells of toxic waste [101-103].

Glucocorticoids play an important role in the cellular stress response and in the maintenance of homeostasis. Glucocorticoids are secreted by the adrenal glands and bind to their receptor (GR), which binds to DNA at the GRE to mediate gene transcription [53]. Glucocorticoids may also work with other transcription factors including HIF-1α and NF-κB to modulate cellular stress responses, inflammation, and hypoxic gene expression [53, 104]. Thus, complex interactions with HIF-1α or NF-κB, or other transcription factors may determine the strength of a GR response to cell signals. These same mechanisms may play a role in the hypoxic induction of luciferase constitutive reporters.

The estrogen receptor (ER) is also in the superfamily of transcription factors that can bind to the StRE; it is activated primarily by estrogen. After ligand binding, the ER is phosphorylated and changes conformation allowing it to bind to DNA to modulate gene expression. The ER can also be activated by cAMP, growth factors, hypoxia, and other steroid hormone receptors, such as T3R, and works with a number of co-factors that affect its activity [8, 105-106]. These cofactors include CBP, ARNT, and HIF-1α. ERα has been shown to work with HIF-1 at the VEGF promoter to regulate transcription [8,
54]. ER-α may also interact with the PI3K/Akt pathway to increase VEGF production [8, 52, 54].

The three consensus sequences found in the promoter regions of the three luciferase constitutive reporters all have the potential to be up-regulated during hypoxia. One or more of these sequences may be responsible for the induction of the luciferase constitutive reporters in hypoxia. Future studies are needed to determine the cause or causes of the hypoxic induction of the luciferase constitutive reporters and if any other experimental treatment has the same effects.
FUTURE STUDIES

NF-κB and the ER are particularly appealing targets for examination of the mechanism of luciferase constitutive reporter induction by hypoxia. Exposure to hypoxia causes the generation of reactive oxygen species and causes the cell considerable stress. NF-κB up-regulation in hypoxia as part of the cell's survival response would explain the large induction of luciferase constitutive reporters. Additionally, the number of NF-κB binding sites in each of the luciferase constitutive reporters is closely correlated to the level of hypoxic induction observed. Varying levels of NF-κB induction in response to hypoxia may explain the differences in luciferase constitutive reporter induction seen in different cell types. All tests should be repeated in a broad range of cell types and using some of the other constitutive reporters, such as lacZ or pEGFP, to identify those most susceptible to error.

In order to begin to elucidate the contribution of transcription factors other than HIF-1 in the hypoxic induction of luciferase constitutive reporters, luciferase constitutive reporter induction would be tested using the split transfection assay and treatment with exogenous hormones or transcription factors, similar to the tests performed for HIF-1. Luciferase constitutive reporters would be tested for induction over a vehicle control after treatment with retinoic acid, to test the retinoic acid receptor family; glucocorticoids, to test GR; estrogen, to test the ER; and exogenous NF-κB to test for NF-κB induction. NF-κB induction of luciferase constitutive reporters can also be tested using an NF-κB specific inhibitor, such as SN50. If NF-κB is responsible for the hypoxic induction of the
luciferase constitutive reporters, then treatment with the inhibitor would abolish the induction of the luciferase constitutive reporters when cells are exposed to hypoxia. Deletional analysis studies can also be used to determine the cause of hypoxic induction of the luciferase constitutive reporters. Once the induction of the luciferase constitutive reporters is ablated through deletion the deleted sequences for each reporter can be analyzed for consensus sequences. The deleted sequences for each luciferase constitutive reporter can also be compared to determine if the same factor is responsible for the hypoxic induction of all three luciferase constitutive reporters.

It is possible that no one factor is responsible for the hypoxic induction of luciferase constitutive reporters. If, however, a factor is identified, deletional analysis may identify the exact location of the responsible binding site and its potential for modification to a non-reactive site. Elucidating the mechanism behind the hypoxic induction will aid scientists who utilize luciferase assays in the design and interpretation of experiments and may lead to better, more reliable constructs for use in future experiments.

The absence of HIF-2α in Rcho-1 trophoblasts also requires much more study. It would be interesting to reexamine the transcriptional activity of HIF-2 in Rcho-1 trophoblasts using a HIF-2 specific reporter such as OCT4 or others that may be identified in the future [107]. It would also be interesting to silence HIF-1α using sh-or si-RNA. Once stable knock-outs have been cloned, the cells can be examined for alterations in differentiation and in their responses to hypoxia using the five endpoints of differentiation as discussed previously. At the same time, the cells can be evaluated for HIF-2α induction. If HIF-2α expression increases in knock-out cells it will support the
theory that HIF-1 and HIF-2 have redundant functions during development and only diverge in adult tissues. The endpoints of differentiation should then be monitored closely for changes that would indicate any differences in function between the homologs.

It is possible that HIF-2α will not be up-regulated after silencing of HIF-1α, indicating that HIF-2α plays no role in the differentiation of Rcho-1 trophoblasts. Further, the functions of HIF-1α during differentiation can be more fully examined without having to consider the contributions of HIF-2α. If no HIF-2α is present after knock-out of HIF-1α, changes in endpoints can be examined to characterize the exact role of HIF-1α in controlling Rcho-1 trophoblast differentiation. Studies examining trophoblast differentiation with sh/si RNA for HIF-1α may also lead to the discovery of other proteins that play a role in certain aspects of differentiation that are sensitive to hypoxia but HIF-1 independent.
CONCLUSIONS

The complex role of HIF-1α and HIF-2α in the development of the placenta has not been fully elucidated. Though other studies have shown a lack of HIF-2α activity in murine embryonic stem cells, it remains necessary to determine at what level HIF-2α is regulated in Rcho-1 trophoblasts. Preliminary studies showed mRNA expression at all levels of hypoxia, supporting other studies. Though no protein was detected in nuclear extracts of Rcho-1 cells cultured in hypoxia, further studies will be needed to clarify the expression and activity of HIF-2α in Rcho-1 trophoblasts and what role, if any, it plays in differentiation.

The finding that the promoter regions of the luciferase constitutive reporters contain a myriad of consensus sequences has broad implications. A number of stressors and treatments have the potential to induce these promoters, making them unsuitable as control reporters in a variety of situations. Previous studies have reported activation of constitutive reporters in response to steroid treatment, stimulation with lipopolysaccharide, GATA transcription factors, and stress-activated MAP protein kinases [65-69, 71-73]. Several other plasmids that are commonly used as control reporters for transfection studies are also under the control of CMV or SV40 promoters, including the enhanced green fluorescent protein (EGFP)-N1 plasmid, and the lacl plasmid [39-40, 43]. These reporters also have the potential for induction by experimental treatments and should also be used cautiously as controls to normalize for transfection efficiency. The mechanisms of hypoxic induction have not been fully
characterized and may include elements not discussed here. Therefore, it is vitally important to use the split transfection assay to normalize for transfection efficiency when using luciferase assays in hypoxia or when using treatments that may induce a stress or hormone response.
REFERENCES


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73. Sulentic CE, Kang JS, Na YJ & Kaminski NE 2004 Interactions at a dioxin responsive element (DRE) and an overlapping kappaB site within the hs4


