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Hypoxia Inducible Factor 1 Alpha (HIF-1a): A Major Regulator of Placental Development

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HYPOXIA INDUCIBLE FACTOR 1 ALPHA (HIF-1α):

A MAJOR REGULATOR OF PLACENTAL DEVELOPMENT

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

By

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B.S., Wright State University, 2011

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Renee Elizabeth Albers ENTITLED HYPOXIA INDUCIBLE FACTOR 1 ALPHA (HIF-1α): A MAJOR REGULATOR OF PLACENTAL DEVELOPMENT BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Albers, Renee Elizabeth. M.S., Microbiology and Immunology Program, Wright State University, 2013. Hypoxia Inducible Factor 1 Alpha (HIF-1α): A Major Regulator of Placental Development.

Hypoxia-inducible factor-1 alpha (HIF-1α) is a critical component of the cellular oxygen-sensing machinery and is essential for placental formation and embryonic survival. In this study, we promoted prolonged expression of HIF-1α, by using a form that is insensitive to oxygen, denoted as CA-HIF-1α. In order to have continual placental specific expression of the CA-HIF-1α, lentiviral infection was performed on embryos at the blastocyst stage of development and transferred into pseudo-pregnant mothers. Placental analysis was performed via *in situ* hybridization on embryonic day 14.5 (E14.5) to determine the effects of CA-HIF-1α prolonged expression. Data indicate that prolonged activity of CA-HIF-1α restricted to trophoblast cells in the mouse placenta results in the inability of cells to advance from their progenitor states, failure of the placenta to organize properly, and failure of trophoblasts to remodel the maternal arteries. Since HIF-1α has the ability to cause developmental placental disruption, its regulation in the placenta could be key in multiple pregnancy-associated disorders such as pre-eclampsia and intrauterine growth restriction (IUGR).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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I. INTRODUCTION

Placental importance

The placenta is an essential transient organ that is formed during mammalian pregnancy. This highly organized organ allows for transport of nutrients and wastes between the mother and the developing fetus. In order to maintain a healthy pregnancy, the placenta needs to remodel maternal arteries to facilitate blood flow. Loss of the placental organization or ability to remodel maternal arteries leads to a reduction in placental function and disorders such as pre-eclampsia [1-5].

Pre-eclampsia

Pre-eclampsia is a pregnancy related disorder that occurs in 5-7% of all births in the United States and is a leading cause of maternal mortality in the world [2,6,7]. Some of the characteristics of pre-eclampsia include maternal hypertension and proteinuria during pregnancy, with these symptoms rapidly diminishing after delivery or termination of the pregnancy [2,6,7]. Pre-eclampsia also leads to premature delivery and low birth weight babies as well as the possibility of fetal death [2,6,7]. Placentas from pre-eclamptic pregnancies exhibit a lack of maternal artery invasion [2,6,7]. In normal pregnancies, the maternal endothelial cells are replaced by invasive trophoblasts in a process known as remodeling [8,9]. The process of maternal artery remodeling is essential to establish appropriate blood flow and promote proper placental development

[4,9]. Due to the placental defects associated with pre-eclampsia, it is important to study placental development.

Placental specific gene transfer

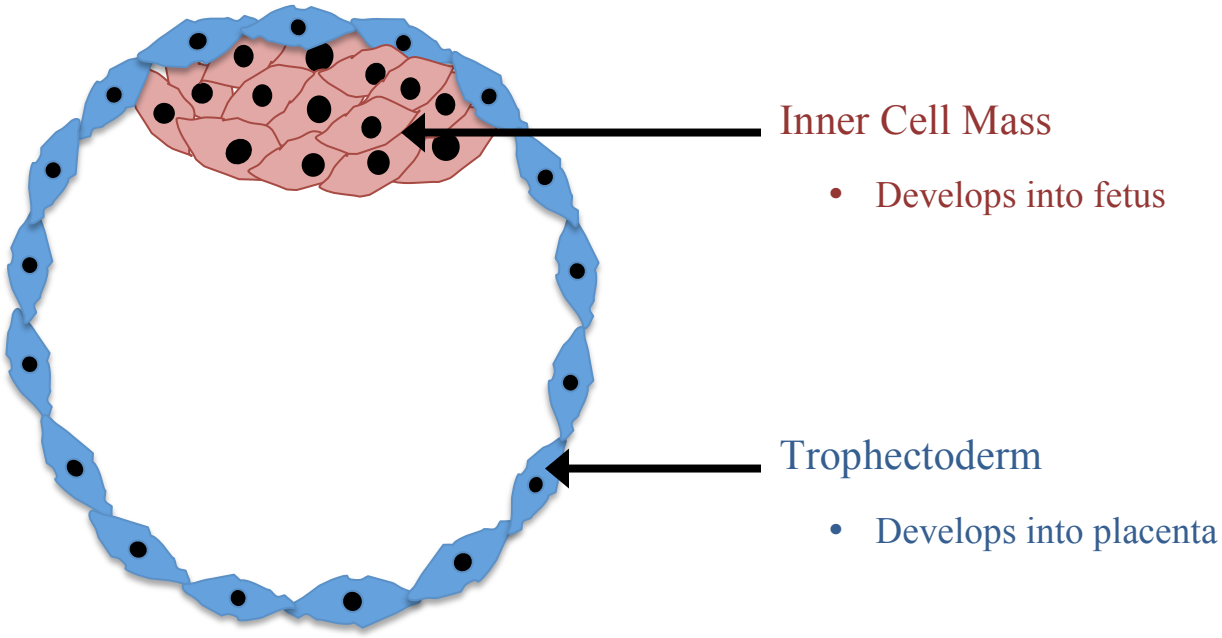
The placenta is formed from a specific set of cells that arise during embryonic development. The embryo progresses through may different stages of development after fertilization, from the two-cell stage to the blastocyst, before implantation. At the blastocyst stage of embryonic development, it is clear that there are two cell types [10-12]. The first cell type at the blastocyst stage, which gives rise to the fetus, is known as the inner cell mass (ICM) [10-12]. The second cell type that is apparent at the blastocyst stage is the trophectoderm, and it develops into the placenta [10-12]. The trophectoderm at the blastocyst stage consists of a single cell layer that surrounds the inner cell mass (Figure 1). In order to obtain stable, placental-specific, expression of a gene, the pre-implantation blastocyst can be infected with lentivirus containing the gene of interest [11-13]. The blastocysts, with only its trophectoderm infected, can then be transferred into a pseudo-pregnant female (foster mom) to facilitate the implantation of the embryo. *Blastocyst infection and then transfer into the pseudo-pregnant female allows for infection of only the placenta but neither the mother nor the fetus, which is essential for placental specific gene studies*.

Placental organization

Figure 4. Blastocyst organization.

The blastocyst consists of two cell types: the inner cell mass and trophectoderm. The inner cell mass gives rise to the developing fetus (red). The trophectoderm is a single cell layer forming the outside of the blastocysts that gives rise to the placenta (blue).

Figure 1.

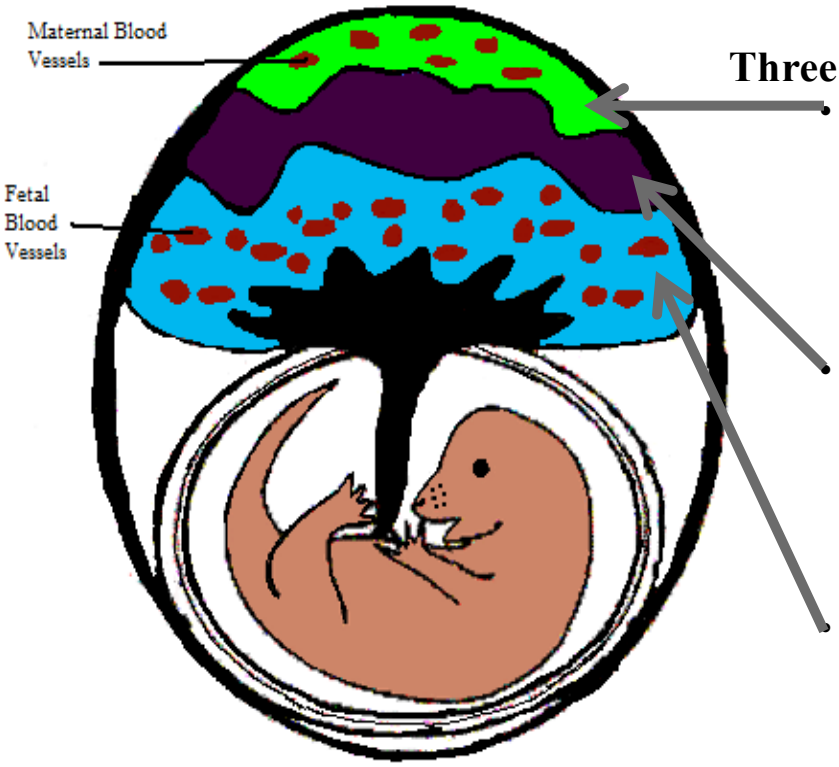

Inner Cell Mass
• Develops into fetus
Trophectoderm
• Develops into placenta

The rodent placenta has analogous structure and function to the human placenta, and has been used as a model system for human placental development [12,14,15]. The rodent placenta consists of three cell lineages: the labrynthine, spongiotrophoblast, and giant cell layers (Figure 2). The labrynthine layer is closest to the fetus and is important in transport of nutrients and wastes between the mother and developing fetus [14]. One of the markers used to identify the labrynthine cell lineage is Glial cell missing 1 (*Gcm-1*) [16,17]. The spongiotrophoblast layer lies between the labrynthine and giant cell layers. The spongiotrophoblast layer regulates exchange between the mother and fetus and acts as a stem cell layer for the giant cells [12]. The marker used for the spongiotrophoblasts layer is trophoblast specific protein alpha (*Tpbpa/4311*) [8]. The giant cell layer, which is closest to the mother, is responsible for invasion and remodeling maternal arteries during implantation [12]. The giant cell layer is identified by placental lactogen II (*Pl2*), which identifies all non-invasive giant cells throughout placental development [18]. The giant cell layer also has a cell subset, marked by proliferin (*Plf*), which is responsible for invasion and remodeling of maternal arteries [18]. Other cells that are important to study in order to understand placental organization include progenitors cells and glycogen cells. Progenitor cells are marked by a transcription factor known as *Mash2* (mammalian achaete-scute homologous protein-2) [19,20]. Progenitor cell distribution is a helpful indicator of the placenta's developmental state since the overexpression of progenitor cells indicates an underdeveloped or immature placenta [19,20]. Glycogen cells are important for energy storage in the placenta and are positioned between the giant cell and spongiotrophoblasts cell lineages [21,22].

Figure 5. Organization of the rodent placenta.

The rodent placenta is organized into three cell lineages: giant cells, spongiotrophoblasts, and labrynthine cells. Each cell type exhibits lineage-specific markers as indicated. Figure modified from formerly published data [23].

Figure 2.



Three layers of trophoblast cells:

- Giant cell layer
  - Invasion of maternal blood vessels
  - Lineage specific markers: **Pl2, Plf**
- Spongiotrophoblast layer
  - Regulation of exchange and source of stem cells
  - Lineage specific marker: **Tpbpa**
- Labrynthine layer
  - For nutrient transport
  - Lineage specific marker: **Gcm-1**

Placental oxygen and development

Differing oxygen levels in the placenta have been implicated in playing an important role in placental development. In the placenta, there is an oxygen gradient in which conditions are very low, near 2% oxygen, during the early stages of development and oxygen levels are near 12% at the maternal arteries [3,24]. During the early stages of placental development when oxygen levels are low, trophoblast cell differentiation is inhibited [25-29]. When trophoblast cells are exposed to higher oxygen concentrations (10-20%), placental differentiation occurs [24,26]. One gene shown to be important in placental cell differentiation is hypoxia-inducible factor 1 alpha (HIF-1α) [4,27,30,31].

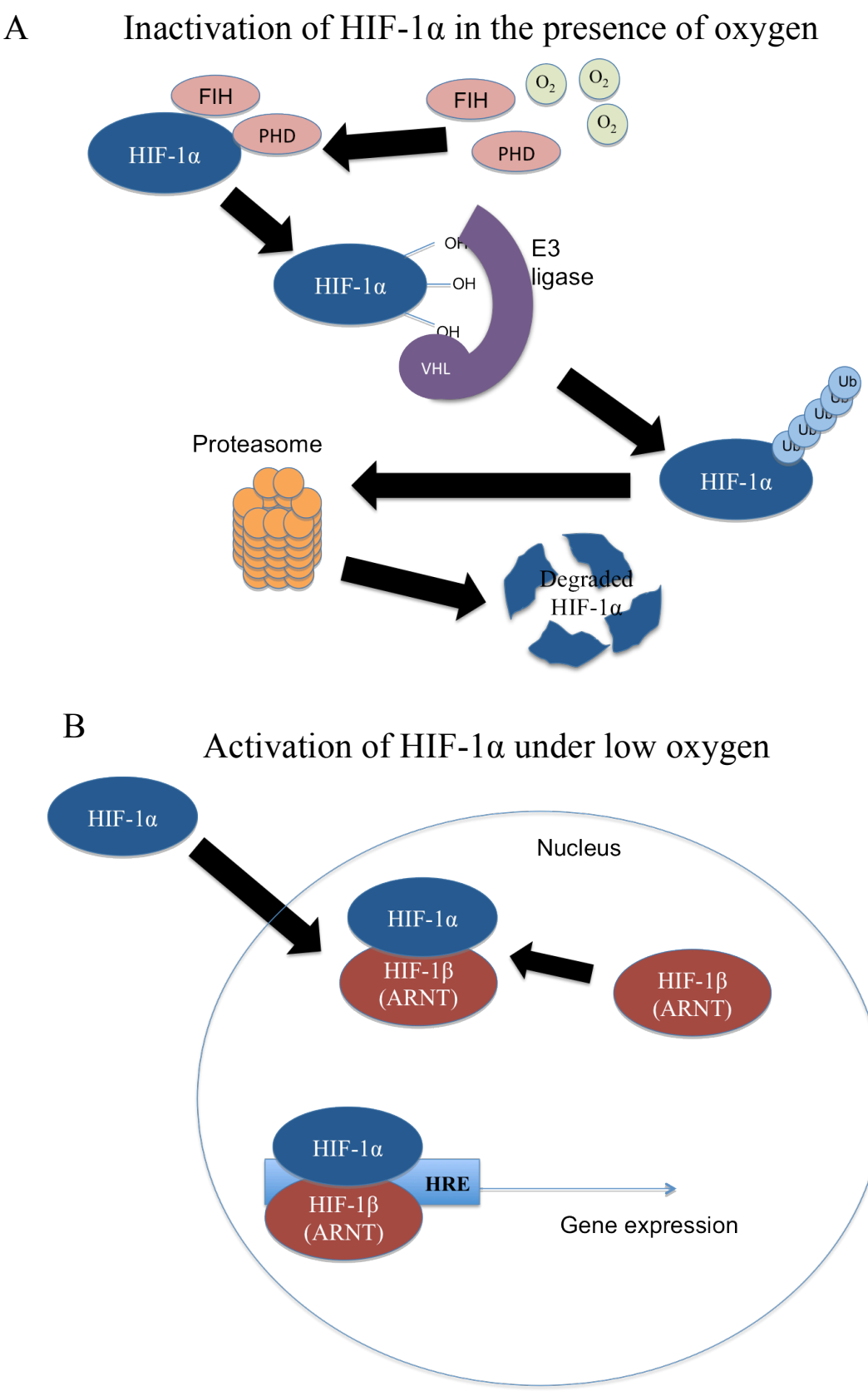
Hypoxia inducible factor 1 alpha (HIF-1α)

HIF-1α is an oxygen-sensing gene in which protein levels are high when oxygen levels are low [32-35]. HIF-1α protein expression is dependent on oxygen levels because it has three sites that become hydroxylated in the presence of oxygen, causing it to be inactive and signaling it toward proteasomal degradation [33,36,37]. The two proline residues on HIF-1α are hydroxylated by prolyl hydroxylase domain proteins (PHD) and the asparagine residue is hydroxylated by an asparaginyl hydroxylase known as factor inhibiting HIF-1α (FIH) in the presence of oxygen [36,37]. Once hydroxylated by PHD, the von Hippel-Lindau region of an E3 ligase recognizes HIF-1α and causes its ubiquination, targeting HIF-1α toward proteasomal degradation [33,37] (Figure 3A). In conditions with low oxygen, HIF-1α protein levels increase and HIF-1α translocates into the nucleus, which allows for dimerization with the constitutively active HIF-1β subunit,

Figure 6. HIF-1 activation and inactivation.

(A) Schematic of how HIF-1α is degraded in the presence of oxygen. (B) Schematic showing how HIF-1α is active in low oxygen conditions and causes gene transcription.

Figure 3.


A
Inactivation of HIF-1α in the presence of oxygen
FIH
PHD
HIF-1α
FIH
PHD
O2
O2
O2
HIF-1α
OH
OH
OH
VHL
E3
ligase
Ub
HIF-1α
Proteasome
Degraded
HIF-1α
B
Activation of HIF-1α under low oxygen
HIF-1α
Nucleus
HIF-1α
HIF-1β
(ARNT)
HIF-1β
(ARNT)
HIF-1α
HRE
HIF-1β
(ARNT)
Gene expression

also known as aryl hydrocarbon receptor nuclear translocator (ARNT) [37,38]. The newly formed heterodimeric protein acts as a transcription factor, regulating genes associated with cell survival, such as placental growth factor (PlGF), and angiogenesis, such as vascular endothelial growth factor (VEGF) [27,33,34,39] (Figure 3B). Former studies have shown that low levels of HIF-1α promote cell differentiation and high levels of HIF-1α promote cell proliferation [27,33,40,41]. In order to create an oxygen insensitive form of HIF-1α that would always promote cell proliferation, the three hydroxylation sites of the protein could be mutated to prevent the hydroxylation [37]. This would allow the concentration of HIF-1α to build and cause gene transcription even in the presence of oxygen, in this stud the oxygen insensitive form of HIF-1α is denoted as CA-HIF-1α (Figure 4).

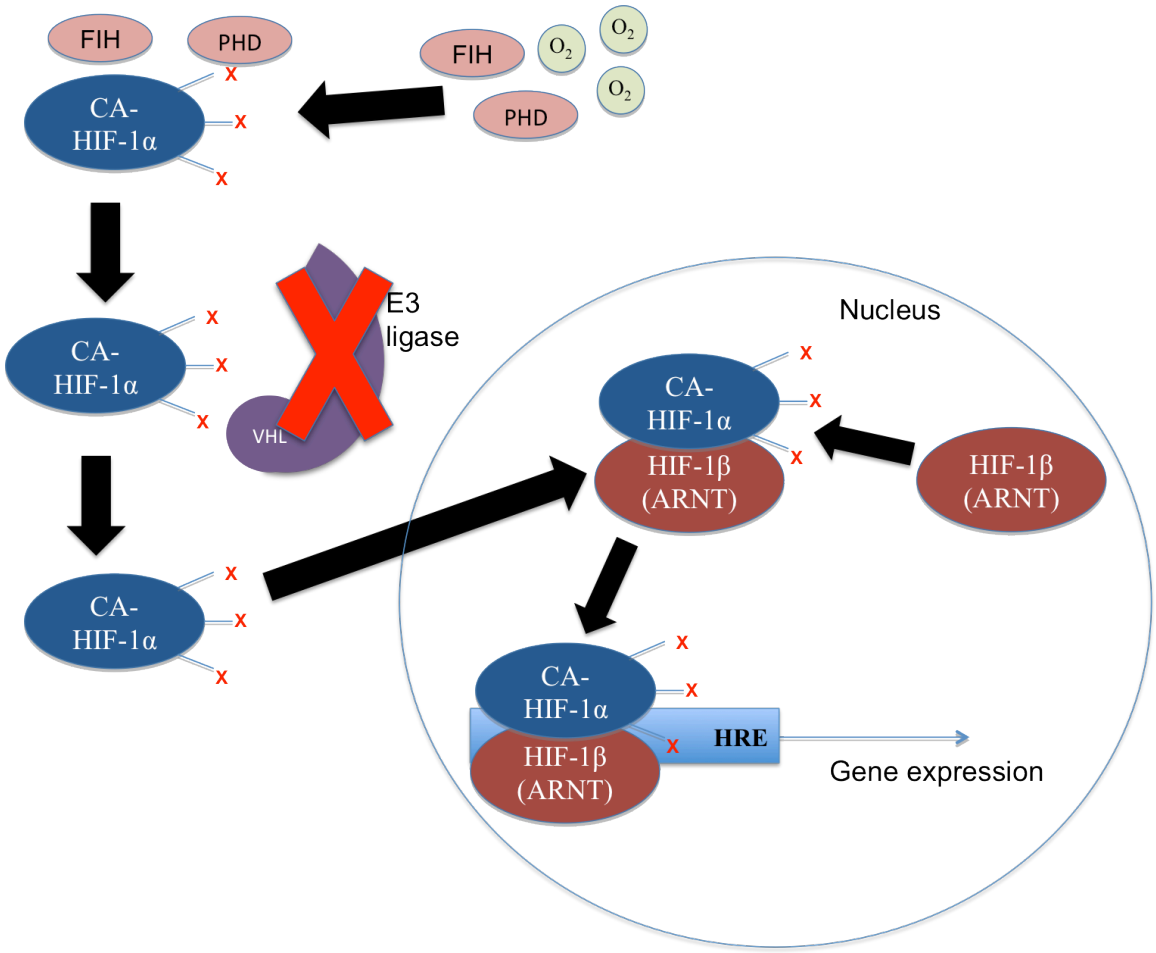
HIF-1α related gene knock-out

Both knock-out models and human placental studies highlight the importance of HIF-1α during embryonic and placental development. Both HIF-1α and HIF-1β (ARNT) knock-outs have proven to be embryo-lethal [28,42-44]. Knocking-out the von Hippel-Lindau (VHL) gene, which is important in recognizing the hydroxylated HIF-1α to signal it toward proteasomal degradation, leads to embryonic lethality due to defective placental vascularization [45]. Knock-outs of PHD-2, the primary form of PHD responsible for hydroxylation of HIF-1α, have also proven to be embryonic lethal [46]. Another gene knock-out study of interest is the study of catechol-O-methlytransferase (COMT), an enzyme involved in metabolism of 2-methoxyestradiol (2-ME), which is known to

Figure 4. Oxygen insensitive form of HIF-1α, CA- HIF-1α, is always active.

(**A**) Schematic of how CA-HIF-1α is able to be active even in the presence of oxygen.

Figure 4.


FIH
PHD
CA-
HIF-1α
FIH
O_2
O_2
PHD
O_2
E3
ligase
VHL
Nucleus
HIF-1β
(ARNT)
HIF-1β
(ARNT)
HRE
Gene expression

suppress HIF-1α in vivo [47]. COMT knock-out mice, which have low 2-ME levels and therefore high levels of HIF-1α, exhibit characteristics similar to pre-eclamptic women [47]. Human placental studies also support the importance of HIF-1α regulation for embryonic and placental development. Placentas analyzed from pre-eclamptic human pregnancies have shown an increase in HIF-1α protein expression [30,31]. Therefore, regulation of HIF-1α is important during embryonic development either in the embryo, in the placenta, or both; therefore, a lack of its regulation could lead to pregnancy-associated disorders.

Summary

Correct placental formation is essential for mammalian pregnancy. Oxygen levels in the placenta have proven to be key in regulation of placental development. Specifically, HIF-1α has been proven to be an important regulator of trophoblast differentiation and proliferation and looks to be important in placental development. In this study, we prolonged the mRNA expression of placental HIF-1α, by using a form HIF-1α, denoted as CA-HIF-1α, that is insensitive to oxygen, in order to determine its importance in embryonic and placental development. Placental-specific, prolonged mRNA expression of HIF-1α was achieved by lentiviral infection of embryos at the blastocyst stage of development, allowing for stable gene expression in the placenta without infection of the mother or fetus. Placental sections were analyzed via *in situ* hybridization to analyze placental organization and development. Our results show that placental-specific, prolonged HIF-1α expression leads to abnormal placental

development, suggesting that regulation by HIF-1α plays a critical role in placental formation. Therefore, deregulation of HIF-1α could lead to pregnancy associated disorders or early termination.

II. MATERIALS AND METHODS

Materials

The plasmid construct pc3-HIF-1α3XSDM was generously provided by Dr. Christina Warnecke of Universität Erlangen-Nürnberg, Germany [26]. Restriction enzymes and buffers used for ligation experiments were purchased from Invitrogen (Carlsbad, CA). The 293FT cell line and Virapower packaging mix was purchased from Invitrogen Corp (Carlsbad, CA). Metafectene transfection reagent was obtained from Biontex (Planegg, Germany) The 5X PEG-it virus precipitation solution was purchased from System Biosciences (Mountain View, CA). HIV-1 p24 Antigen ELISA 2.0 kit used for viral titering was obtained from ZeptoMetrix Corporation (Buffalo, NY). C57Bl/6 male and female mice, ICR female mice, and vasectomized ICR male mice were purchased from Harlan Laboratories (Indianapolis, IN) or Taconic Farms Inc. (Germantown, NY). Pregnant Mare's Serum gonadotropin (PMS), Human Chorionic Gonadotropin (HCG), and mineral oil were acquired from SIGMA-Aldrich Co. (St. Louis, MO). EmbryoMax M2 Media and EmbryoMax KSOM embryo culture media were obtained from Millipore (Billerica, MA.) The Non-Surgical Embryo Transfer Device (NSET) was purchased from ParaTechs Corporation (Lexington, KY). Vectashield mounting media was obtained from Vecotor laboratories (Burlingame, CA).

Cloning and Cell Culture

In order to create the lentiviral construct (pLv) containing the HIF-1α3XSDM gene (oxygen insensitive form of HIF-1α) under the constitutive Cytomegalovirus

(CMV) promoter; the pc3-HIF-1α3XSDM construct and pLB2V5 (a construct generated by replacing the Green Fluorescent Protein (GFP) gene of pLv-CMV-GFP-V5 with a multiple cloning site) were digested using BamHI and ApaI restriction enzymes. DNA fragments for ligation were isolated and the HIF-1α3XSDM gene was then ligated into the pLB2V5 using the compatible BamHI and ApaI ends and T4 DNA ligase to generate pLB2V5-CA-HIF-1α. The ligation constructs were transformed into One Shot Stbl3 Chemically Competent Cells (Invitrogen, Carlsbad, CA). Transformed constructs were selected by ampicillin resistance (50μg/mL). DNA was isolated from bacterial cells and the construct was confirmed by differential restriction digestion and sequencing (Cleveland Genomics, Cleveland, OH).

293FT cells were cultured in DMEM/High glucose (Thermo Scientific, Waltham, MA), 10% heat-inactivated fetal bovine serum (Biowest, Kansas City, MO), 1% antibiotic-antimycotic (Thermo Scientific, Waltham, MA), 1 mM sodium pyruvate (Mediatech, Inc., Manassas, VA), 2 mM glutaGRO Supplement (Mediatech, Inc., Manassas, VA), 0.1 mM NEAA Mixture (Lonza, Walkersville, MD) and 500 μg/mL G418 (InvivoGen, San Diego, CA). Cos7 cells were cultured in DMEM/High glucose (Thermo Scientific, Waltham, MA), 10% heat inactivated fetal bovine serum (Biowest, Kansas City, MO), and 1% antibiotic-antimycotic (Thermo Scientific, Waltham, MA). Rcho-1 cells were cultured as previously described [26,48]. All cell types were passaged at 80-90% confluence.

Western blotting (Kashmira Kulkarni unpublished methods)

Nuclear extracts for analysis of HIF-1a were completed by using CellLytic NuCLEAR Extraction Kit following the manufacturer's specifications. Cells were scraped and collected in chilled 1xPBS and followed by lysis via use of a hypotonic lysis solution. Nuclear extracts were isolated by treatment with specified solutions and differential centrifugation. Proteins were used for Western blot analysis or stored at -80^{o}C until further use. Bradford method was used to determine nuclear extract concentration and samples were analyzed spectrophotometrically at 595nm [49]. The concentration of protein in each sample was determined using a Bovine serum albumin standard protein curve. Nuclear extracts (12.5-50 μg protein) were reduced using 1x reducing sample buffer (62.5 μM Tris pH 6.8, 2% SDS, 12.5% glycerol, 2.5% Beta-mercaptoethanol and 0.1% of 2.5% bromophenol blue in ethanol [50]. Samples were boiled at 95^{o}C for 10 minutes and separated on 8%-10% SDS-polyacrylamide gel. Precision plus protein standard dual colored markers were used to determine the molecular weights (BioRad, Berkeley, California). Proteins separated by SDS-PAGE electrophoresis were transferred to PVDF (polyvinylidene fluoride) membrane in transfer buffer (25mM Tris pH 8.0, 192 mM glycine and 20% methanol overnight at 100mA constant current [26,51]. Ponceau S staining was used to confirm protein transfer. The membrane was incubated with blocking buffer (5% Non- fat milk, 60 mM Tris base, 204 mM NaCl, 0.025% Tween 20, pH 7.4) for 1hr at room temperature on a rocker. Subsequently, the blot was incubated with primary antibody at 4^{o}C overnight or 1hr at room temperature on a rocker. The membrane was washed three times with 1x PBS containing 0.05% Tween 20, and was incubated with secondary antibody for 1hr while rocking. After 1hr incubation, the membrane was washed three times with 0.05% Tween

20 in 1XPBS [26,51]. Specific protein was identified with ECL chemiluminiscence reagent (Amersham).

Luciferase assay (Kashmira Kulkarni unpulblished methods)

To determine Hypoxia Response Element (HRE) reporter activity, cos7 cells were transiently transfected with 0.2 μg pRL-SV40-promoter constitutive reporter plasmid, 1μg PGK1-HRE luciferase reporter plasmid, 1μg of plasmid (either pLB2V5, pLB2V5-CA-HIF-1α, or pc3-HIF-1α3XSDM) and 5μl Metafectene reagent. Media was changed 18hrs post transfection. Twenty-four hours post media change, luciferase reporter activity was determined by using the Dual Luciferase Assay system (Promega) according to the manufacturer's protocol. Results show the fold-increase in HRE activity in samples cultured at ambient oxygen.

Lentiviral production

293FT cells were transfected with 9 μg of the plasmid containing the gene of interest, either pLB2V5-CA-HIF-1α or pLv-CMV-GFP-V5, and optimized Virapower packaging mix, including 4.6 μg pLP1, 1.8 μg pLP2, and 2.52 μg pLp-VSVG, (Invitrogen, Carlsbad CA) using 50 μl Metafectene transfection reagent (Biontex, Planegg, Germany). Media was changed at 24hrs post transfection and virus was collected at 72hrs after media change. Virus containing media was centrifuged for 20 minutes at 3,000rpm. The supernatant was stored at –80^{o}C and later used for infection.

Lentiviral concentration and titering

Viral supernatant was mixed with 5X PEG-it virus precipitation solution (System Biosciences, Mountain View, CA) and incubated overnight at 4°C. The mix was then centrifuged at 3,000 rpm for 30 min at room temperature. The supernatant was discarded and pellet was obtained. The pellet was re-suspended in 1XPBS and Polybrene (Millipore, Billerica, MA) at a final concentration of 10 μg/ml. The re-suspended pellet was used for infections of blastocysts. Titering for viral concentrations was completed with concentrated virus following manufacturer's specifications using HIV-1 p24 Antigen ELISA 2.0 (ZeptoMetrix Corporation, Buffalo, NY). Briefly, samples and standards were prepared and added to pre-washed microplate. The microplate was covered and incubated for 3 to 24 hours at 37 °C and then washed before adding HIV-1 Detector Antibody. The microplate was covered and was incubated at 37 °C for 1 hour. The microplate was washed, substrate was added, and incubated at room temperature for 30 min. The reaction was stopped by adding Stop Solution and plate was read within 15 min at 450 nm on a plate reader. For infection, LB2V5-CA-HIF-1α or Lv-CMV-GFP-V5 lentivirus was concentrated to a final infective concentration of 6,800 ng/ml or 1,200 ng/mL, respectively.

Blastocyst Isolation and Culture

Four to five week old (14-16 gram) C57Bl/6 female mice were super-ovulated by injection of 5 International Units (IU) PMSg, Pregnant Mare's Serum gonadotropin (SIGMA-Aldrich, St. Louis, MO), and 47 hours later injected with 5IU HCG, Human

chorionic gonadotropin (SIGMA-Aldrich, St. Louis, MO). Females were allowed to mate with male C57Bl/6 mice immediately after HCG injection and plug checked at 0.5 days post copulation (d.p.c.). At 1.5 d.p.c., super-ovulated females were CO_2 euthanized and the oviducts were removed for 2-cell collection. The oviducts were shredded in EmbryoMax M2 Media with phenol red for collection (Millipore, Billerica, MA). Microdrop cultures were setup 18-24 hours before use by covering 20 µl drops of EmbryoMax KSOM embryo culture media (Millipore, Billerica, MA) with mineral oil (SIGMA-Aldrich, St. Louis, MO) in a 35 mm cell culture dish and incubated at 37.5^{o}C at 5.5% CO_2. Isolated embryos were placed in Microdrop culture of EmbryoMax KCL Simplex Optimization Medium (KSOM) and incubated at 37.5^{o}C and 5.5% CO_2 until further use.

Blastocysts infection

Concentrated virus that was re-suspended in 1XPBS and Polybrene mixture was placed in a 35 mm plate and covered with approximately 2 ml of mineral oil to create virus microdrop culture. Virus microdrop culture was allowed to incubate for 30 minutes at 37.5^{o}C and 5.5% CO_2 prior to treatment of blastocysts. Blastocysts at day E3.5 were treated with acidic Tyrode's solution (SIGMA, St. Louis, MO), to remove the zona pellucida, the protective protein coat that surrounds the embryo and prevents viral infection. Blastocysts were subsequently washed three to four times in EmbryoMax M2 medium, and transferred into virus microdrop cultures and incubated for 4 to 6 hours at 37.5^{o}C and 5.5% CO_2. Post infection, blastocysts were washed in EmbryoMax M2

embryo culture media to remove virus. Embryos were combined into one drop of EmbryoMax M2 medium and were used for embryo transfer.

Embryo transfer

Eight to ten week old Imprinting Control Region (ICR) mice (23-33 grams), were allowed to mate with vasectomized ICR males overnight to produce pseudo-pregnant females. Females were plug checked at 0.5 d.p.c., and females positive for copulation were used for embryo transfer. Post-lentiviral infection blastocysts were washed in EmbryoMax M2 medium and 10-20 embryos were transferred into a 2.5 d.p.c. pseudo-pregnant ICR mouse via an NSET Device, Non-Surgical Embryo Transfer Device (ParaTechs Corporation, Lexington, KY).

Immunocytochemistry (Kashmira Kulkarni unpulblished methods)

Placenta and embryo sections were hydrated with 3 washes in 1xPBS for 5 minutes each. Hydration was followed by permeabilization treatment with TritonX-100 (0.2% TX-100 in 1xPBS). Permeabilization was followed by quenching with glycine (1% in 1xPBS). Sodium borohydrate (NaBH4 0.1% in 1xPBS) treatment for was used to quench autoflourescence. The tissues were then blocked in blocking solution (Goat serum 10%, tween 0.05% in 1xPBS) for 1hr at room temperature. Primary antibody (V5-topo at 1:500) was applied overnight in 1% blocking solution at 4^{o}C overnight in a humidified chamber. The following day sections were washed with 1xPBS and

secondary antibody (Alexa Fluor 594 Goat anti rabbit, at 1:1000) was applied in 1% blocking solution for 1hr at room temperature in a dark box. Sections were washed with 1xPBS and treated with Hoeschst stain (1ug/ml) for 5 minutes at room temperature followed by 3 washes with PBS. Using Vectashield mounting media, glass cover slips were mounted on the plate surface, and staining was observed by fluorescence microscopy.

Probes

Digoxigenin (DIG)-labeled riboprobes were prepared according to the manufacturers protocol (Roche) (Figure 5). Riboprobes for *Pcdh12, Tpbpa, Gcm1, Prl3b1/Pl2, Prl2c2/Plf,* and *Ascl2/Mash2* have been previously described (Table 1) [16,17, 19,52-56]. Riboprobes for *Pecam-1* were generated from a plasmid that was created in the lab. Briefly, PCR was used to amplify an 811 base pair region corresponding to nucleotides 1784 to 2595 of the *Pecam-1* messenger RNA sequence (NM008816). This fragment was subsequently cloned into pGEM-Teasy (Promega). For detection of *Hif1a*, riboprobe template was amplified by RT-PCR utilizing forward and reverse primers incorporating T3 and T7 RNA polymerase recognition sites, respectively. The primer sequences were: Forward/T3, 5'-AATTAACCCTCACTAAAGGGCAGTCGACACAGCCTCGATA,

Reverse/T7, 5'-TAATACGACTCACTATAGGGTTTGGAGTTTCCGATGAAGG and amplify an amplicon of approximately 670 bp.

Tissue preparation and in situ hybridization

Conceptuses for *in situ* hybridization were dissected at E14.5 in cold 1xPBS after CO_2 asphyxiation, fixed overnight in 4% paraformaldehyde (PFA) in 1XPBS at 4°C, embedded in paraffin and sectioned (7 μm) as previously described [53,57]. For in situ hybridization, sections underwent de-paraffinization in xylene followed by rehydration through a graded ethanol series ending in PBS. Sections were then post-fixed in 4% PFA, treated with proteinase K to degrade protein and reduce background (15 mg/ml for 5 minutes at room temperature). Then, sections were acetylated for 10 minutes (acetic anhydride, 0.25%; Sigma) and hybridized with DIG-labeled probes overnight at 65^{o}C. Hybridization buffer contained 1X salts (200mM NaCl, 13 mM Tris, 5 mM sodium phosphate monobasic, 5 mM sodium phosphate dibasic, 5 mM EDTA), 50% formamide, 10% (w/v) dextran sulfate, 1 mg/ml yeast tRNA (Sigma), 1X Denhardt's [1% (w/v) bovine serum albumin, 1% (w/v) Ficoll, 1% (w/v) polyvinylpyrolidine) and cRNA probe (final dilution of 1:2000 from reaction with 1 mg template DNA). Post-hybridization washes were followed by an RNase treatment [400 mM NaCl, 10mM Tris (pH 7.5), 5 mM EDTA, 20 mg/ml RNase A]. After blocking with blocking solution (hybridization buffer lacking cRNA probe), sections were incubated overnight at 4^{o}C in alkaline phosphatase-conjugated, anti-DIG antibody (Sigma) diluted 1:2500 in blocking solution. Sections were washed and signals were detected using alkaline phosphatase immunohistochemistry with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) as the substrate as previously described [53,58]. Sections were counterstained with nuclear fast red.

Figure 5. Diagram of a riboprobe.

Schematic of how *in situ* hybridization works via use of a DIG-labeled riboprobe.

Figure 5.

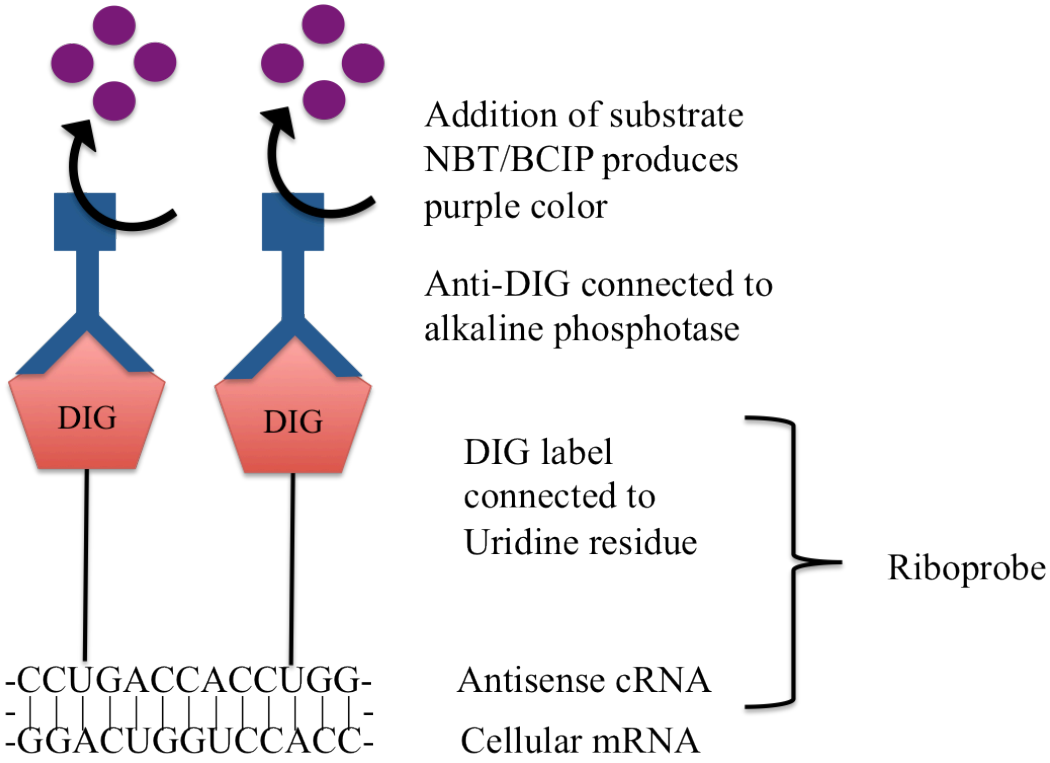

DIG
DIG
-CCUGACCACCUGG-
-GGACUGGUCCACC-
Addition of substrate NBT/BCIP produces purple color
Anti-DIG connected to alkaline phosphotase
DIG label connected to Uridine residue
Antisense cRNA
Cellular mRNA
Riboprobe

Table 1. Genes surveyed by in situ hybridization.

Marker Name	Marker Abbreviation	Cell Type Marked	Marker Citation
Hypoxia inducible factor 1 alpha	*HIF-1α*	N/A	Current Study
Mammalian achaete-scute homologous protein-2	*Mash2*	Progenitor cells	[19]
Glial cell marker-1	*Gcm-1*	Labyrinthine progenitors	[16]
Trophoblast specific protein alpha	*Tbpba*	Spongiotrophoblasts	[52]
Protocadherin-12	*Pcdh12*	Glycogen cells	[53]
Placental lactogen II	*Pl2*	Non-invasive giant cells	[53]
Platelet/endothelial cell adhesion molecule-1	*Pecam-1*	Maternal endothelial cells	Natale (unpublished data)
Proliferin	*Plf*	Invasive giant cells	[54]

Animal use

All animal procedures were performed in accordance with the Wright State University Laboratory Animal Care and Use Committee (LACUC).

III. RESULTS

Placental specific expression

Previous studies have shown the ability to obtain placental specific gene expression via infection at the blastocyst stage, embryonic day 3.5 (E3.5) [11,13]. Infection at the blastocyst stage allows for placental-specific gene transfer because the embryo consists of two cell types, the ICM, which gives rise to the fetus and the trophectoderm, the single-cell outer layer which gives rise to the placenta [10-13]. Lentiviral infection and then embryo transfer at this stage allows for gene expression in the placenta without the mother or fetus expressing the gene of interest [11,13]. Following this method, we infected blastocysts with Lv-CMV-GFP-V5 virus, which used GFP as a reporter gene. We confirmed lentiviral infection of the blastocyst via fluorescent microscopy to detect GFP expression (Figure 6). In order to confirm that the infection was placental specific, fetoplacental units were collected at E10.5 and were visualized for GFP expression via fluorescent microscopy. Only the placenta (P) showed GFP expression, but the decidua (D), maternal tissue, of the mother and the embryo (E) lacked GFP expression (Kashmira Kulkarni, unpublished data, Figure 7). Placental transduction by Lv-CMV-GFP-V5 was confirmed by expression of both GFP and V5 epitope tag since the V5 epitope is located at the end of the GFP reading frame and is followed by a stop codon (Kashmira Kulkarni, unpublished data, Figure 8A). While GFP expression was observed by fluorescent microscopy (Figure 8C), V5 epitope expression (red) was observed throughout the GFP expressing placenta by immunocytochemical analysis (Figure 8D) and co-localization of the GFP expression and V5 staining was

Figure 6. Trophoblast specific GFP expression after infection with Lv-CMV-GFP-V5 at the blastocyst stage.

Mouse blastocyst (E3.5) devoid of zona pellucida was infected with concentrated Lv-CMV-GFP-V5 virus for 4-6 hours at 37.5^{o}C and 5.5% CO_2. (**A**) The bright field image of a post infection. (**B**) GFP expression was assessed post infection via fluorescent microscopy.

Figure 6.

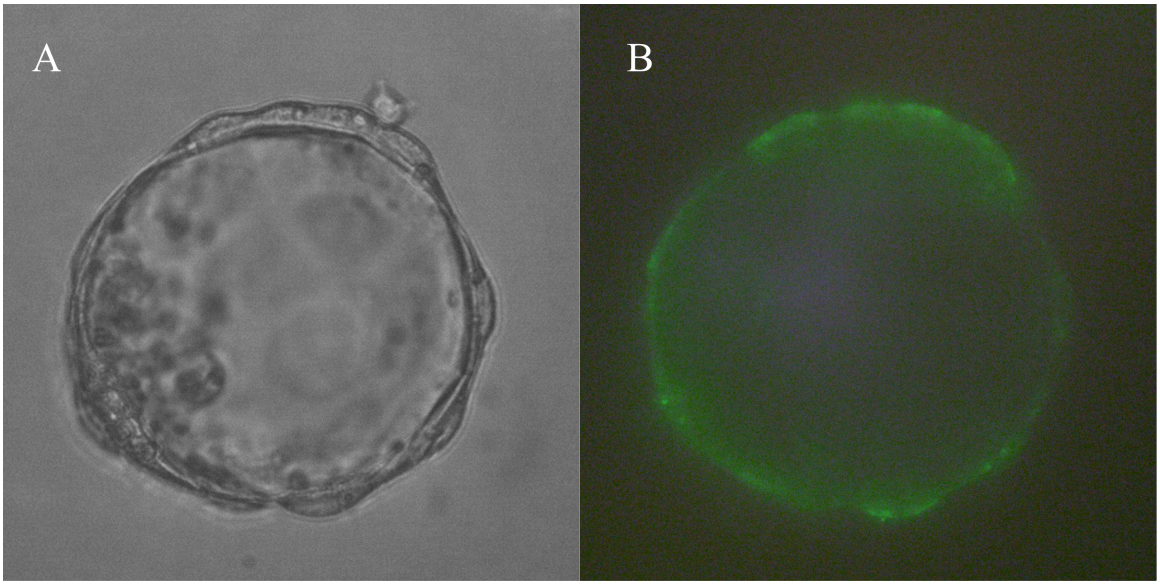
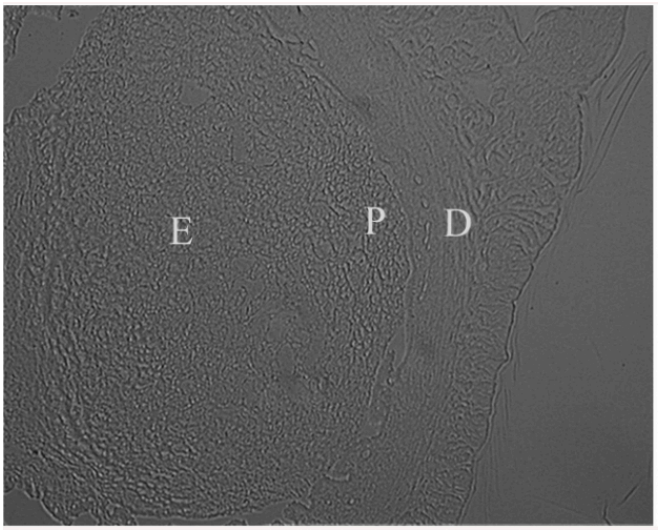


Figure 7. Analysis for placental specific expression of the GFP at E10.5 after infection with Lv-CMV-GFP-V5 at the blastocyst (E3.5) stage of development.

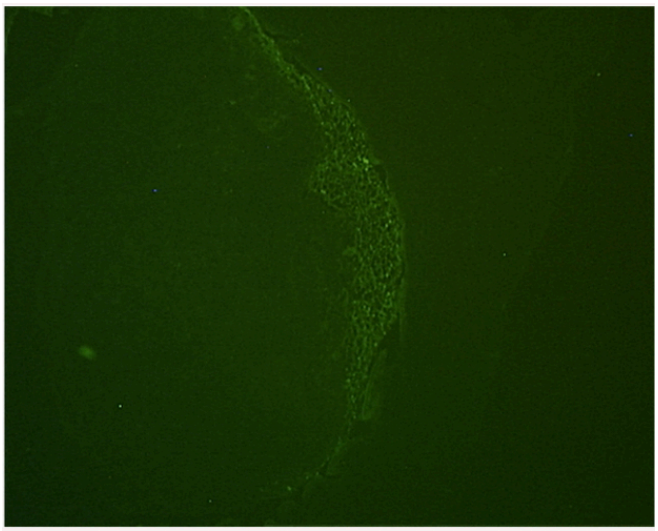
Mouse blastocyst (E3.5) devoid of zona pellucida was infected with concentrated Lv-CMV-GFP-V5 and transferred into pseudo-pregnant mother. Feto-placental unit was collected at E10.5 and was fixed in 4% paraformaldehyde and embedded in O.C.T. Eight micron sections were examined for GFP expression via fluorescent microscopy. **E** indicates the embryo, **P** indicates the placenta, and **D** indicates the maternal decidua. (**A**) Bright field image of the feto-placental unit, (**B**) fluorescent image to analyze GFP expression, (**C**) overlay of bright field and fluorescent images from A and B.

Figure 7.

A



B

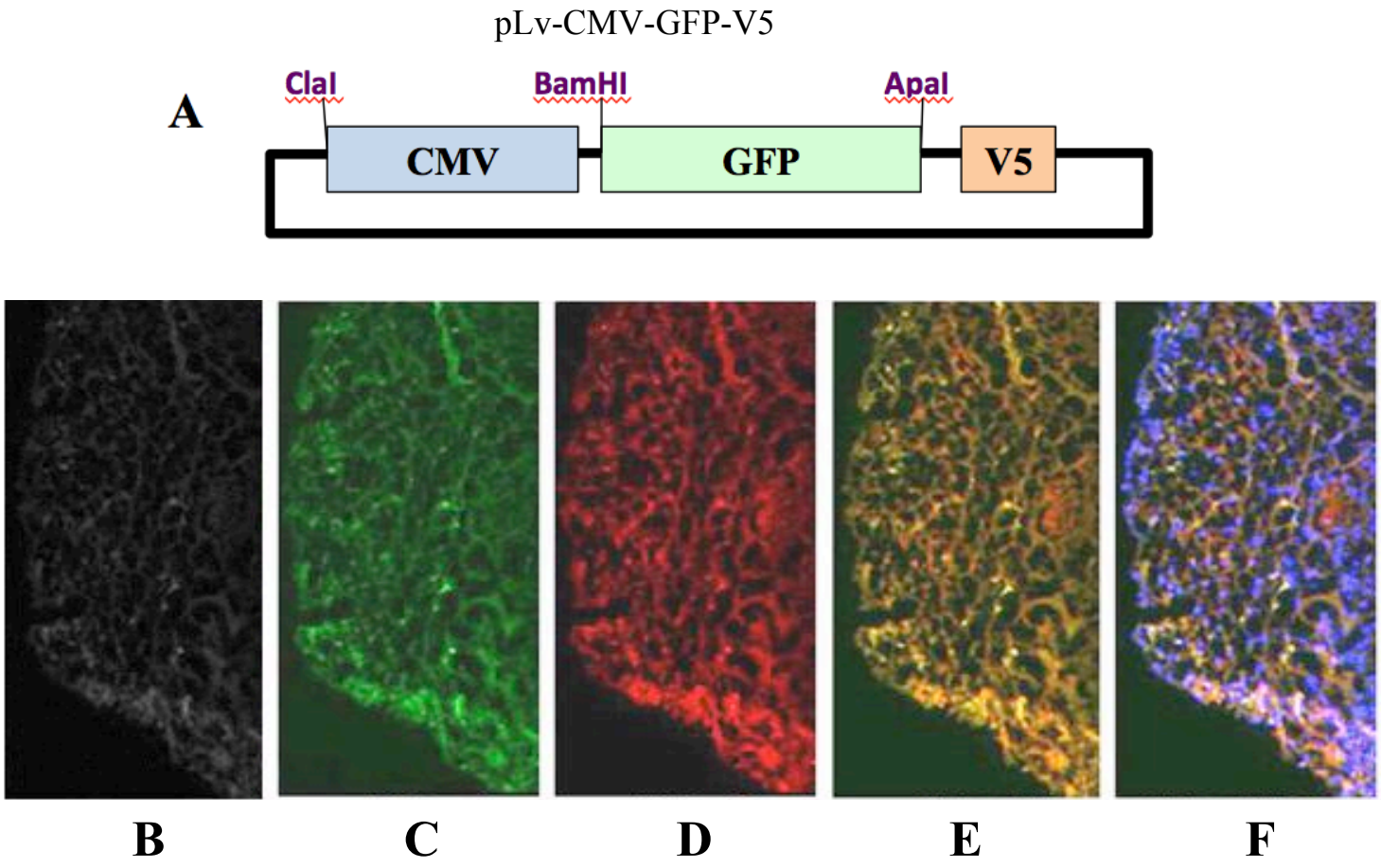


C

Figure 8. Immunohistochemical analysis of V5 epitope tag expression in placenta infected with Lv-CMV-GFP-V5 virus.

Placentas were obtained on embryonic day 12.5 (E12.5) from blastocysts infected with concentrated Lv-CMV-GFP-V5. Tissues were fixed in paraformaldehyde and embedded in O.C.T. Eight micron sections were examined for V5 epitope tag expression by immunocytochemistry analysis. (**A**) Diagram of Lv-CMV-GFP-V5 construct depicting position of V5 epitope tag. (**B**) Bright field image of placenta. (**C**) Fluorescent microscopy image of GFP expression. (**D**) Alexa Fluor 594 secondary antibodies were used to probe for polyclonal antibodies specific to V5 epitope (red). (**E**) Overlay of images **C** and **D**, where yellow color identifies overlap of GFP expression (green) and V5 staining (red). (**F**) Hoechst staining (blue) was performed for nuclear staining on GFP sections stained with anti-V5 epitope tag.

Figure 8.



observed in the pLv-CMV-GFP-V5 infected placenta (Figure 8E). Additionally, Hoechst staining was performed for the visualization of nuclei (Figure 8F).

HIF-1α prolonged expression

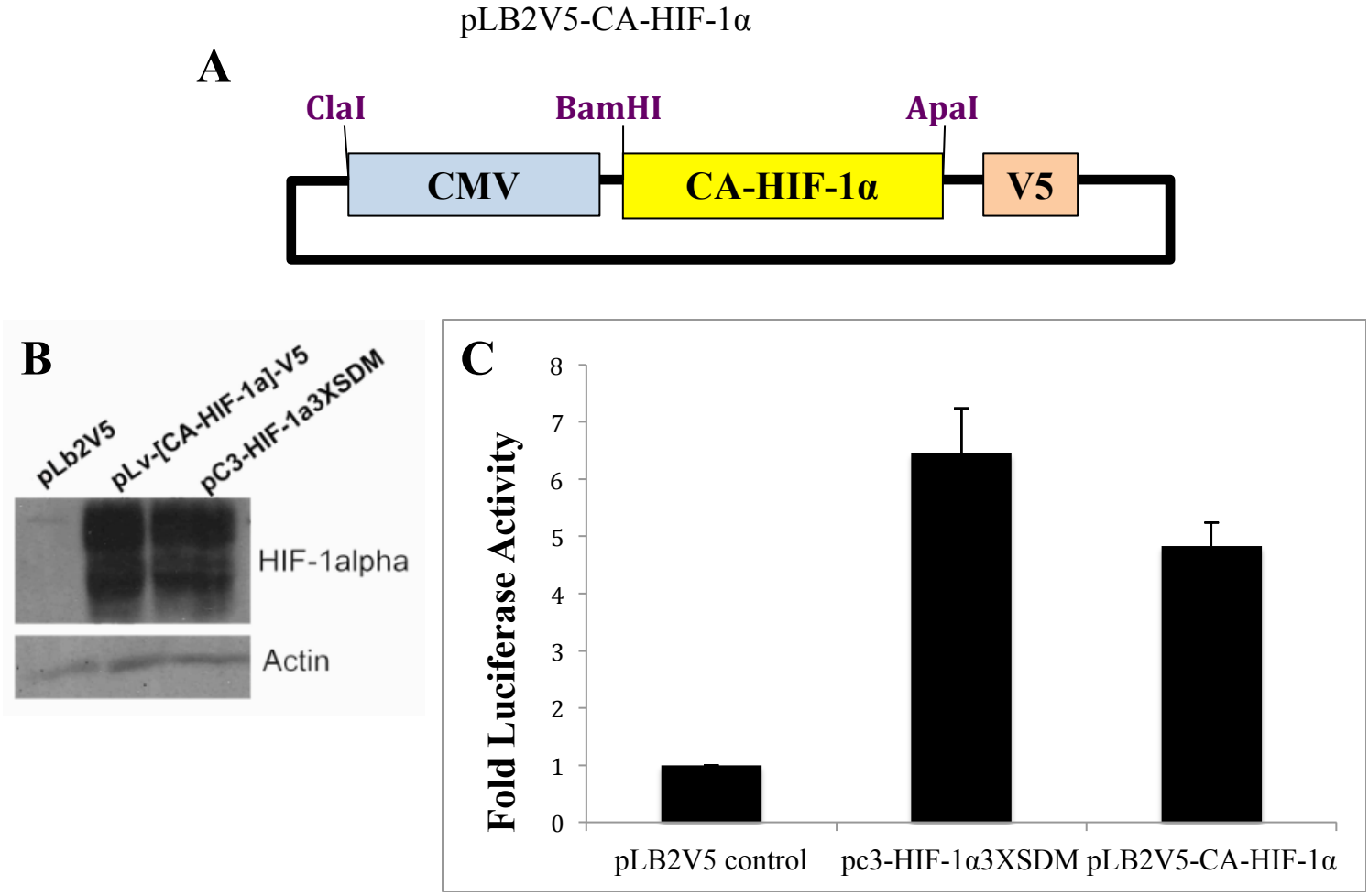
In order to cause prolonged expression of HIF-1α, we generated a lentiviral-based plasmid, denoted as pLB2V5-CA-HIF-1α, encoding HIF-1α protein that is no longer oxygen sensitive due to mutations of amino acids that prevent its hydroxylation and signaling toward proteasomal degradation (Figure 9A) [37]. Western blot analysis was performed on Rcho-1 cells transiently transfected with pLv-CMV-GFP-V5, pLB2V5-CA-HIF-1α, or pc3-HIF-1alpha3XSDM (positive control). Western analysis confirmed increased HIF-1α protein expression by newly formed pLB2V5-CA-HIF-1α construct under ambient oxygen (Figure 9B). To test the ability to bind the hypoxia response element (HRE) that allows for transcriptional activation, the newly formed pLB2V5-CA-HIF-1α and the base plasmids of pLB2V5 (lentiviral backbone) and pc3-HIF-1alpha3XSDM (plasmid containing oxygen independent HIF-1α) were transiently transfected into Cos7 cells. The pLB2V5-CA-HIF-1α and base plasmid pc3-HIF-1alpha3XSDM resulted in activation of the phosphoglycerate kinase-hypoxia responsive element- luciferase reporter (PGK-HRE-luciferase), indicating that CA-HIF-1α was able to bind the hypoxia response element under ambient oxygen conditions (Figure 9C).

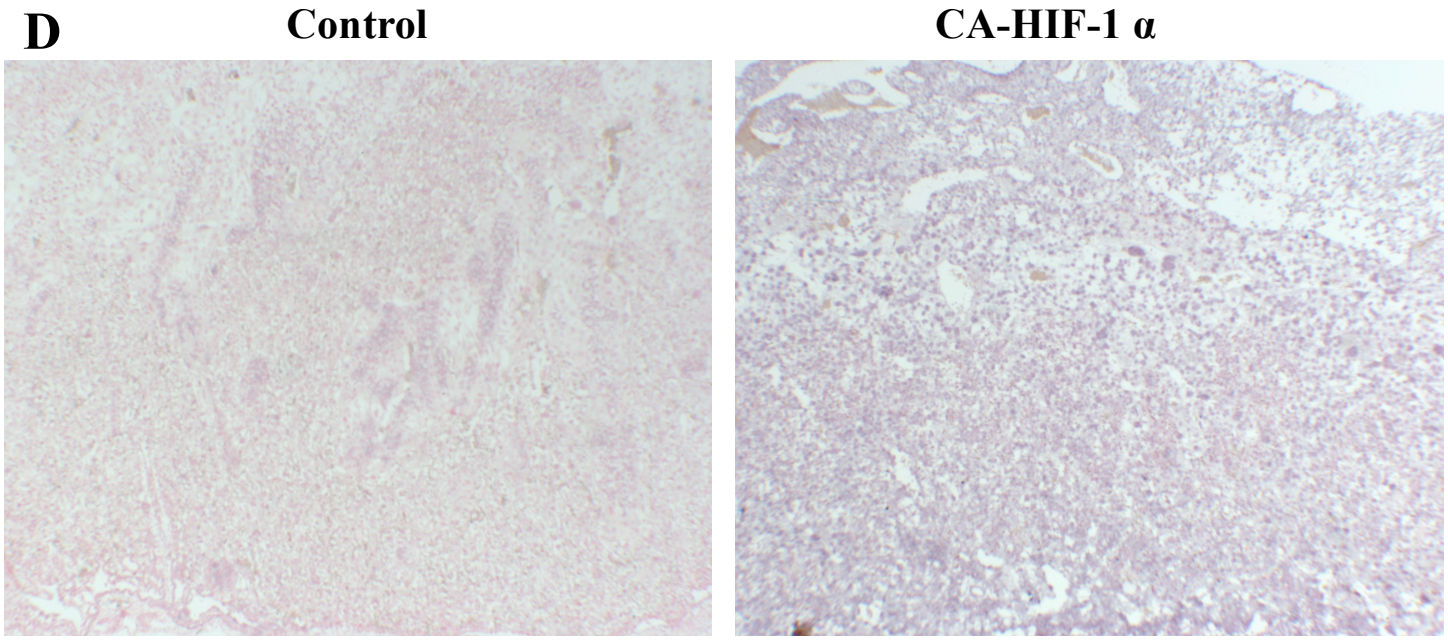
Prolonged expression of CA-HIF-1α *in vivo* was verified via *in situ* hybridization using a HIF-1α riboprobe. Blastocysts, devoid of zona pellucida, infected with either Lv-CMV-GFP-V5 or LB2V5-CA-HIF-1α and transferred into pseudo-pregnant mothers and

Figure 9. HIF-1α3XSDM gene was cloned into lentiviral vector and *in situ* hybridization analysis for prolonged expression of HIF-1α.

HIF-1alpha3XSDM gene was excised from pc3-HIF-1α3XSDM plasmid and ligated into pLB2V5 plasmid using BamHI and ApaI restriction enzymes. (**A**) Diagram of newly formed pLB2V5-CA-HIF-1α plasmid construct, which was confirmed by sequencing. Rcho-1 cell were transiently transfected with pLv-CMV-GFP-V5, pLB2V5-CA-HIF-1α, or pc3-HIF-1alpha3XSDM and Western blot analysis was completed to confirm expression of HIF-1α protein at ambient oxygen (**B**). Cos7 cells were transiently transfected with pLB2V5, pLB2V5-CA-HIF-1α, or pc3-HIF-1alpha3XSDM and HIF-1alpha expression was analyzed under ambient oxygen conditions and PGK-1-HRE reporter activity was determined using Luciferase reporter assay (**C**). (**D**) Placentas from either LB2V5-CA-HIF-1α (right panel) or Lv-CMV-GFP-V5 control (left panel) infected blastocysts were collected at embryonic day 14.5 (E14.5) and fixed in paraformaldehyde and embedded in paraffin. Placental sections were hybridized with DIG-labeled riboprobes specific for *HIF-1α,* denoted by purple staining. DIG-expression was analyzed using alkaline phosphatase immunohistochemistry. Sections were counterstained with nuclear fast red.

Figure 9.


pLB2V5-CA-HIF-1α
A
ClaI
BamHI
ApaI
CMV
CA-HIF-1α
V5
B
pLb2V5
pLv-[CA-HIF-1a]-V5
pC3-HIF-1a3XSDM
HIF-1alpha
Actin
C
Fold Luciferase Activity
0
1
2
3
4
5
6
7
8
pLB2V5 control
pc3-HIF-1α3XSDM
pLB2V5-CA-HIF-1α


D
Control
CA-HIF-1 α

fetuses and placentas were collected at E14.5 for analysis. Placentas infected with LB2V5-CA-HIF-1α (Figure 4B, right) showed much greater HIF-1α expression than those infected with Lv-CMV-GFP-V5 control virus (Figure 9D, left), proving that infection with LB2V5-CA-HIF-1α does prolong the expression of HIF-1α.

To test the functionality of placentas that have prolonged expression of HIF-1α, placental and fetal weights were measured at E14.5 and the placental tissue was analyzed. When comparing both placental and fetal weights of pregnancies from blastocysts infected with control Lv-CMV-GFP-V5 virus or LB2V5-CA-HIF-1α, there is no significant difference in the placental or fetal weights at E14.5 (Figure 10). However, placental analysis via *in situ* hybridization exhibited a drastic change in placental morphology.

Inhibition of differentiation

Prolonged expression of HIF-1α showed a tendency for cells to remain in progenitor states. Progenitor cell distribution was analyzed by *in situ* hybridization of *Mash 2* (Figure 11). *Mash 2* expression is greatly increased in placentas exhibiting prolonged HIF-1α (Figure 11, right) compared to control placentas expressing GFP (Figure 11, left). This evidence shows that prolonged placental HIF-1α is inhibiting trophoblasts from progressing past progenitor cells states. Specific glycogen cell distribution was analyzed via *Pcdh12* (Figure 1). Generally, the prolonged expression HIF-1α placentas (Figure 11, right) showed less organization of glycogen cells when compared to control placentas,

Figure 10. Prolonged HIF-1α gene expression does not affect fetal or placental weight at E14.5 stage in development.

Mouse blastocyst (E3.5) was infected with concentrated pLv-CMV-GFP-V5 or pLB2V5-CA-HIF-1α and transferred into pseudo-pregnant mother. Placentas and fetuses from either pLB2V5-CA-HIF-1α (n= 11 fetuses and placentas) or pLv-CMV-GFP-V5 (n= 7 fetuses and placentas) infected blastocysts were collected at embryonic day 14.5 (E14.5) and weighed immediately after dissection.

Figure 10.

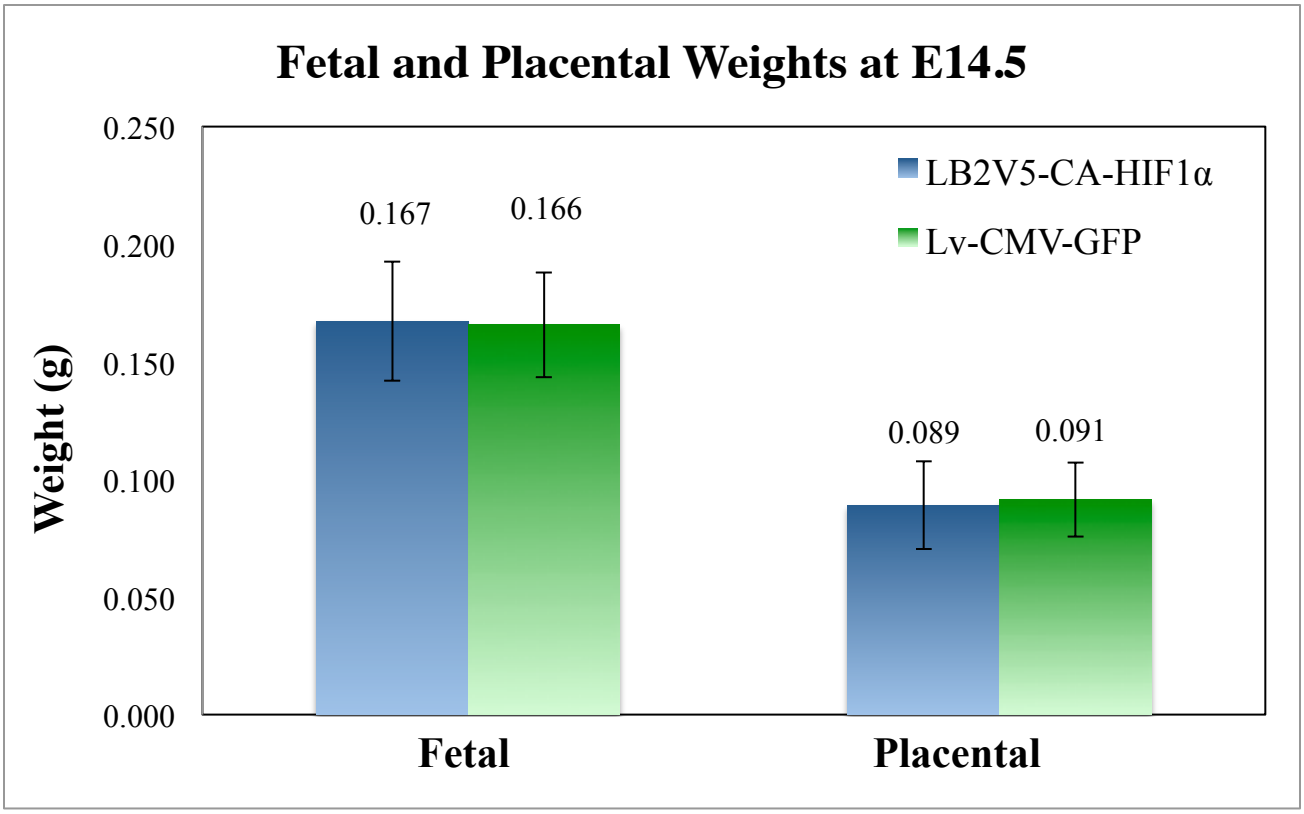
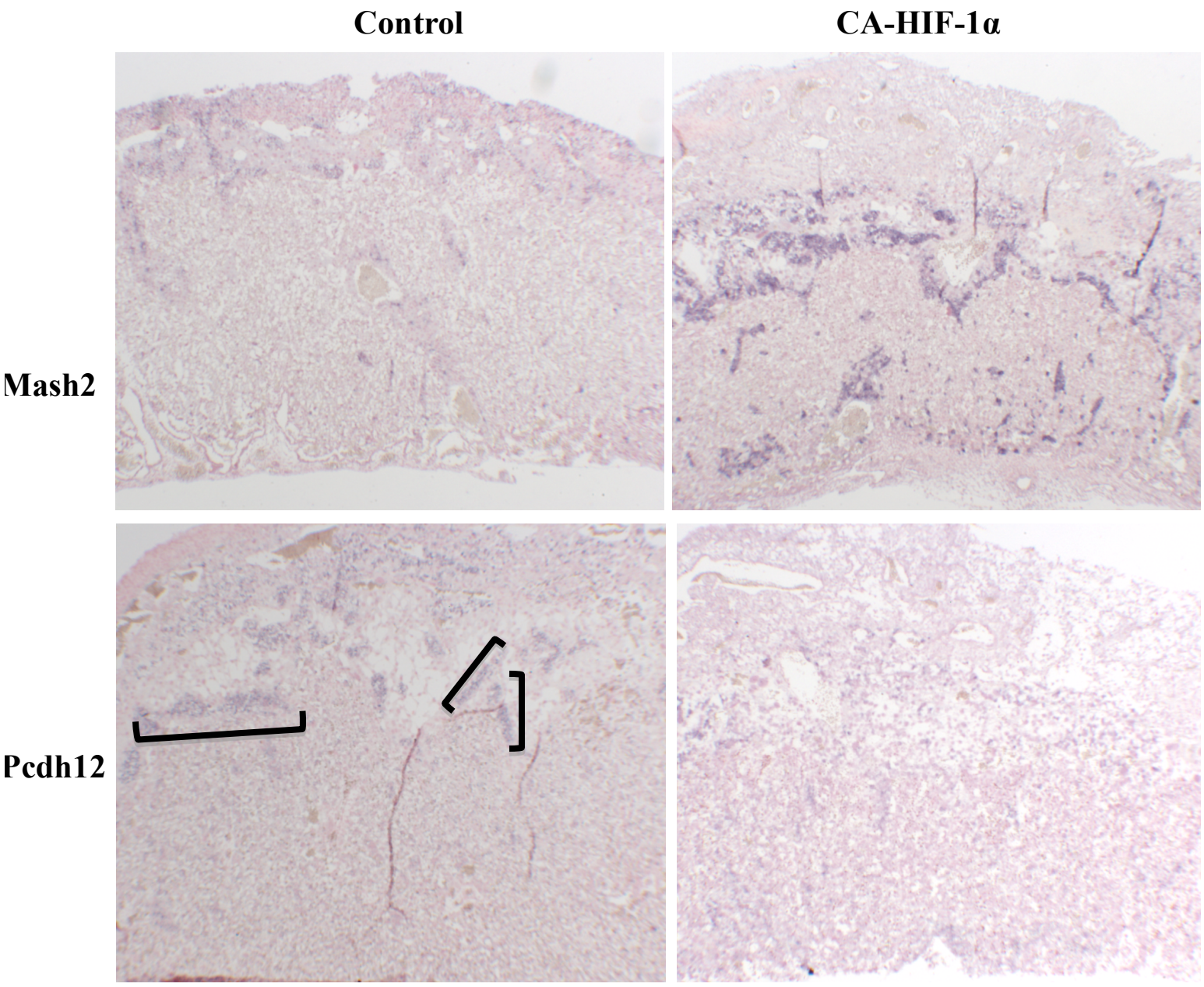

Fetal and Placental Weights at E14.5
0.250
0.200
0.150
0.100
0.050
0.000
Weight (g)
0.167
0.166
0.089
0.091
LB2V5-CA-HIF1α
Lv-CMV-GFP
Fetal
Placental

Figure 11. Prolonged HIF-1α gene expression inhibits placental cells from progressing past their progenitor states.

Placentas and fetuses from either LB2V5-CA-HIF-1α (right) or Lv-CMV-GFP-V5 (left) infected blastocysts were collected at embryonic day 14.5 (E14.5) and fixed in paraformaldehyde and embedded in paraffin. Placental sections were hybridized with DIG-labeled riboprobes specific for *Mash 2 (*progenitor cell marker) or *Pcdh12* (glycogen cell marker), denoted by purple staining. DIG-expression was analyzed using alkaline phosphatase immunohistochemistry. Sections were counterstained with nuclear fast red. Brackets indicate areas with organized glycogen cells.

Figure 11.



which had organized glycogen cells (indicated by brackets) (Figure 11, left). These data indicate a lack of timely and proper differentiation as well as placental disorganization.

Decrease in branching morphogenesis

Analysis of the labrynthine layer was determined by *in situ* hybridization for *Gcm-1* (Figure 12). Upon viewing a gross section of the placentas, *Gcm-1* expression in the HIF-1α placentas is less prevalent and more disorganized than in control placentas (Figure 12). Upon further investigation at higher magnification, punctate expression (indicated by arrows) of *Gcm-1* was observed in the labrynthine layer of HIF-1α placentas, where as a normal banding and distributed expression of *Gcm-1* (indicated by brackets) was seen in the control placentas shown by brackets (Figure 12, bottom). Disorganization and limited, punctate expression of *Gcm-1* is indicative of a lack of branching morphogenesis, as shown by arrows (Figure 12, bottom).

Placental disorganization

To analyze the organization of the spongiotrophoblasts, *in situ* hybridization for *Tpbpa* was assessed. In CA-HIF-1α placentas, the spongiotrophoblast layer is compact and cells in this layer have much less *Tpbpa* expression in comparison to control placentas (Figure 13). These data suggest disorganization of the placenta as a whole and decrease in trophoblast stem cells.

Since the spongiotrophoblast layer suggests placental disorganization, the giant cell layer was also analyzed via *in situ* hybridization for *Pl2* (Figure 13). The number of giant cells

Figure 12. Prolonged HIF-1α gene expression decreases branching morphogenesis.

Placentas and fetuses from either LB2V5-CA-HIF-1α (right) or Lv-CMV-GFP-V5 (left) infected blastocysts were collected at embryonic day 14.5 (E14.5) and fixed in paraformaldehyde and embedded in paraffin. Placental sections were hybridized with DIG-labeled riboprobes specific for *Gcm-1* (lineage specific labyrinthine cell marker for branching morphogenesis), denoted by purple staining. DIG-expression was analyzed using alkaline phosphatase immunohistochemistry. Sections were counterstained with nuclear fast red. (Top) *Gcm-1* at 4X magnification. (Bottom) *Gcm-1* at 20X magnification with brackets indicating banding, distributed expression and arrows indicating punctate expression.

Figure 12.

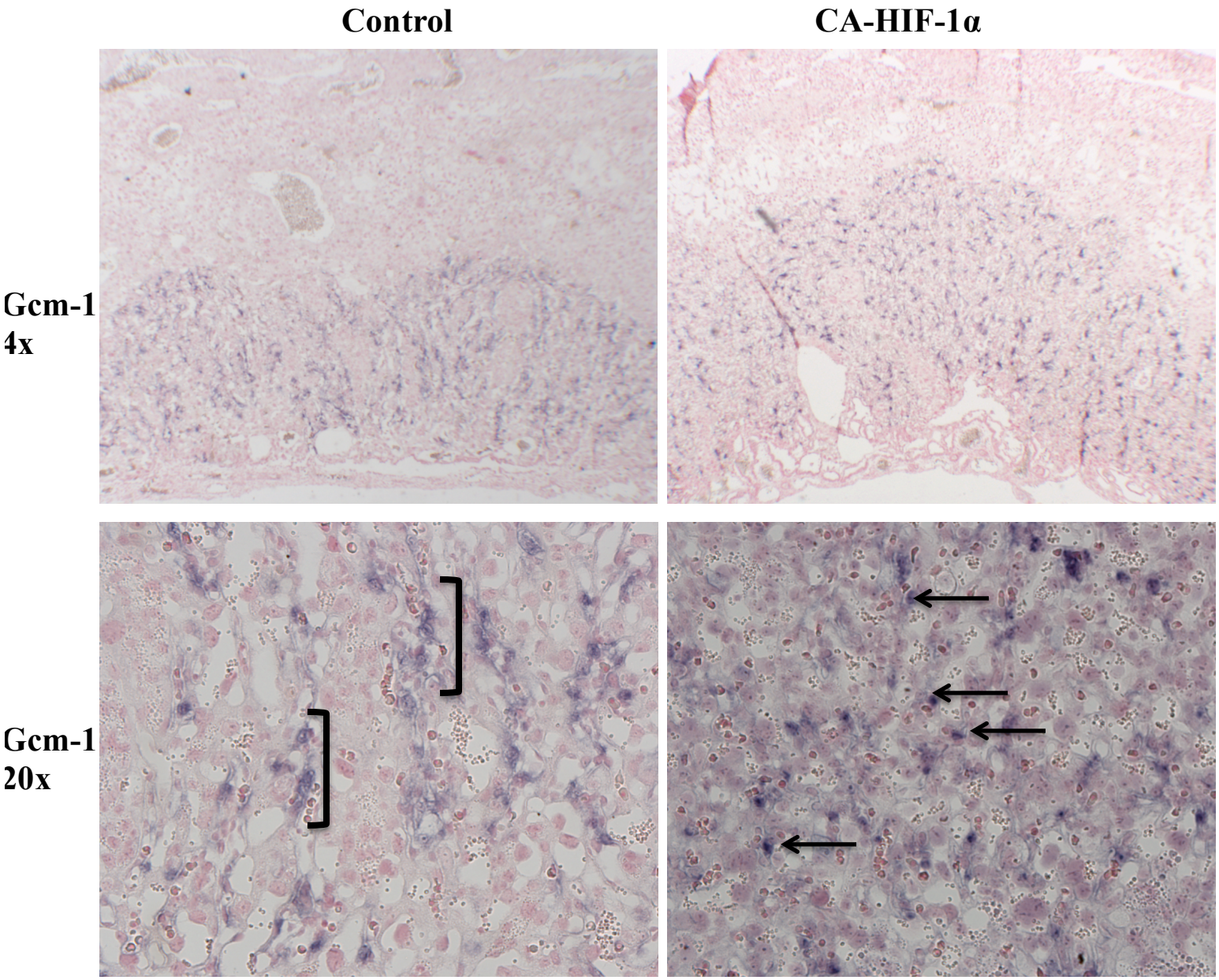
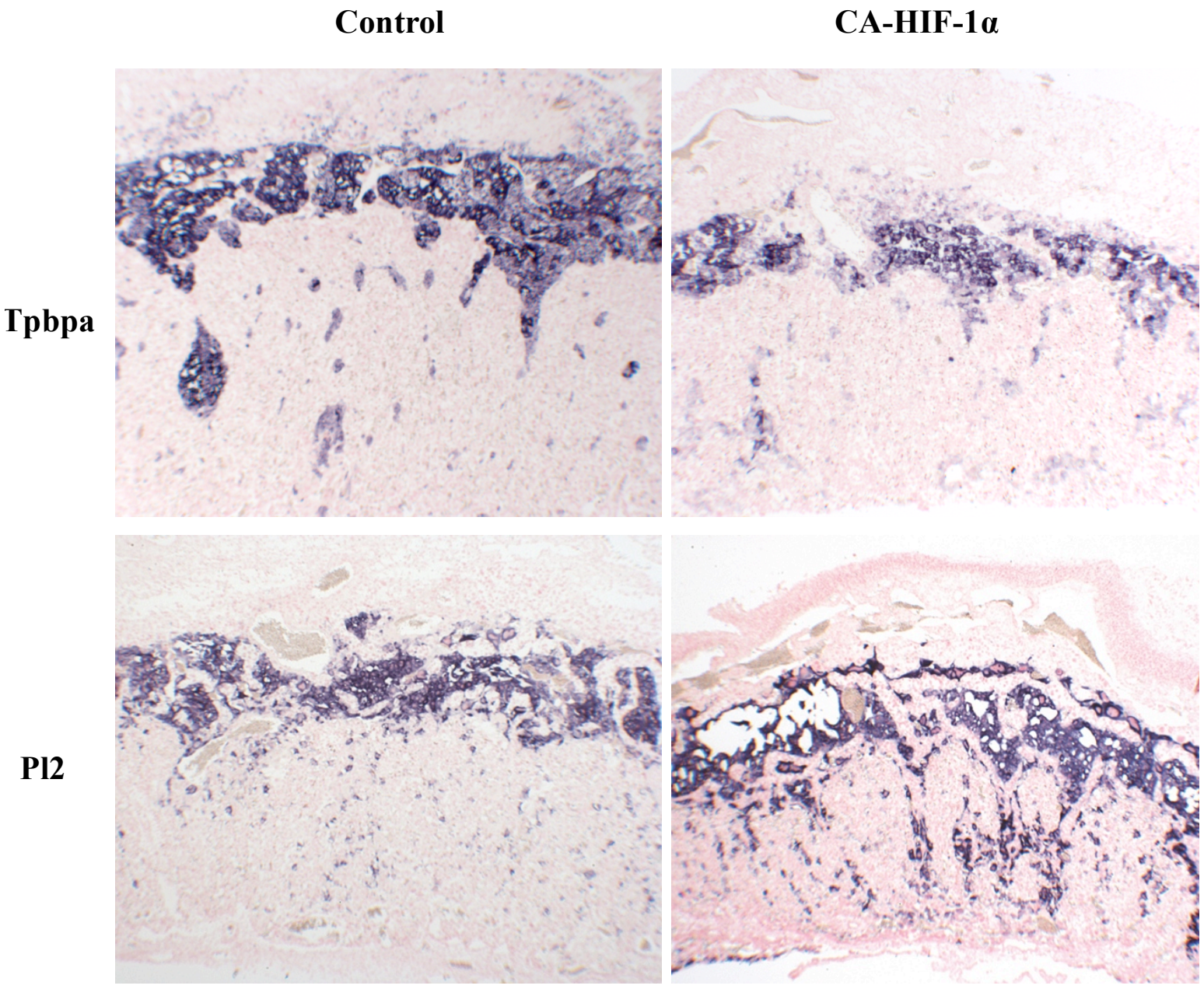


Figure 13. Prolonged HIF-1α gene expression decreases placental organization.

Placentas and fetuses from either LB2V5-CA-HIF-1α (right) or Lv-CMV-GFP-V5 (left) infected blastocysts were collected at embryonic day 14.5 (E14.5) and fixed in paraformaldehyde and embedded in paraffin. Placental sections were hybridized with DIG-labeled riboprobes specific for *Tpbpa (*lineage specific spongiotrophoblasts cell marker) or *Pl2* (lineage specific giant cell marker), denoted by purple staining. DIG-expression was analyzed using alkaline phosphatase immunohistochemistry. Sections were counterstained with nuclear fast red.

Figure 13.



in the HIF-1α placentas (Figure 13, right) was greatly increased when compared to the control placentas. In addition to the increase in giant cells in the HIF-1α placentas, the giant cells were located in areas of the placenta that predominantly contain labrynthine cells and very few giant cells, as seen in the control placenta (Figure 13, left). The increase in giant cells and lack of spongiotrophoblasts may be due to a shuttling of the spongiotrophoblasts stem cells into the giant cell lineage. Together, the giant cell layer and spongiotrophoblast layer demonstrate the lack of organization apparent in the placentas with prolonged mRNA expression of HIF-1α (Figure 13).

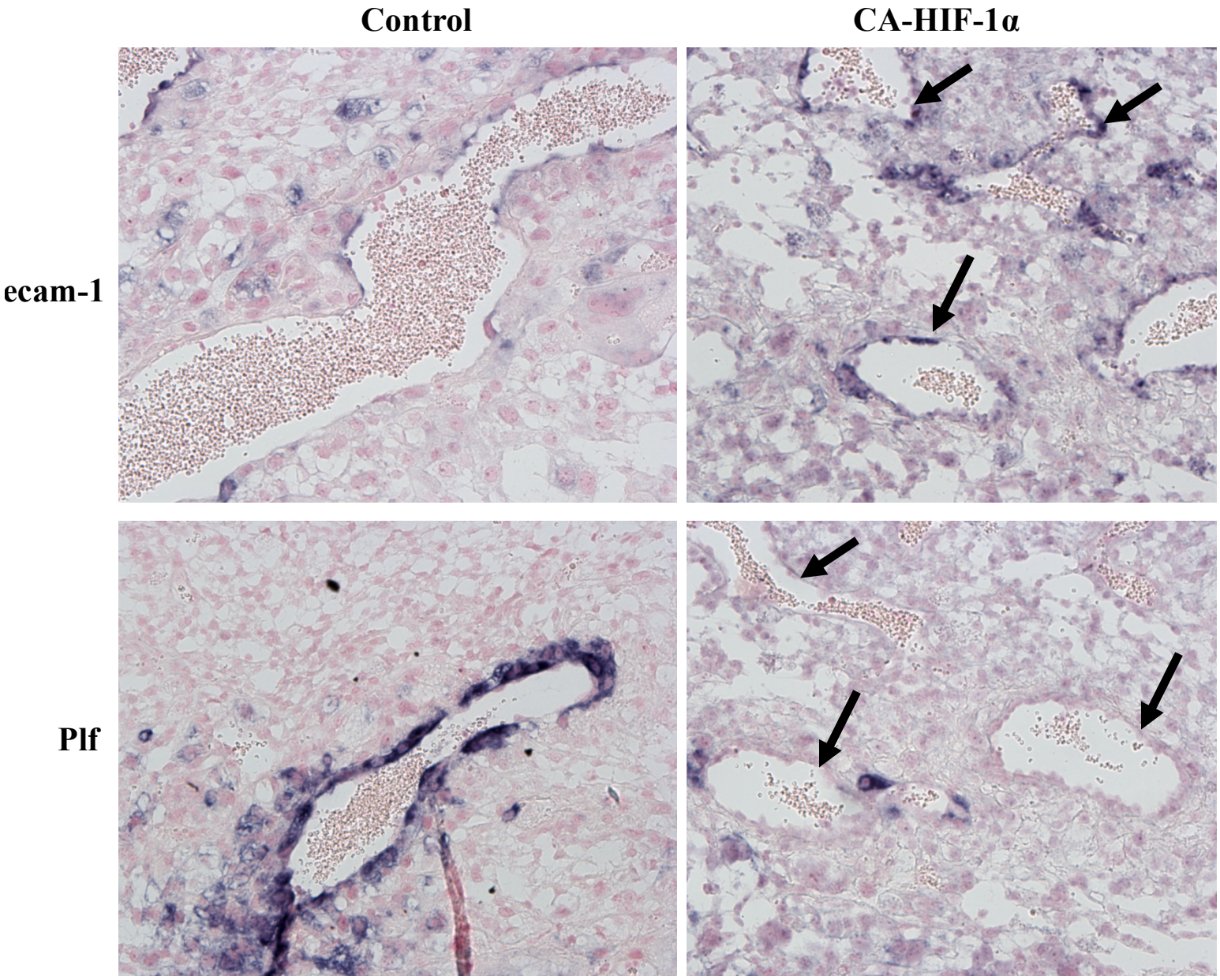
Decrease in maternal artery remodeling

After disorganization of the giant cell layer was confirmed, the functionality of the giant cells was investigated. In order to determine if invasive giant cells were functioning normally and remodeling the maternal arteries, which is an essential step in appropriate placental and embryonic development, placentas were analyzed via *in situ* hybridization for *Pecam-1* (maternal endothelial cell marker), which marks maternal endothelial cells [8,59]. Cells surrounding the maternal arteries (indicated by arrows) in HIF-1α placentas show considerably more *Pecam-1* expression than control placentas, which show very little expression surrounding maternal arteries (Figure 14). The greater expression in HIF-1α placentas suggests a lack of maternal remodeling, but to further investigate maternal remodeling, placentas were analyzed for *Plf* expression, which indicates the presence of invasive giant cells, and is a gene that is not directly under HIF-1α regulation [28]. Placentas with prolonged HIF-1α (Figure 14, right) reveal a lack in *Plf* expression surrounding maternal arteries (cells surrounding arteries are indicated by arrows), where control mice (Figure 14, left) exhibited robust *Plf* expression surrounding

Figure 14. Prolonged HIF-1α gene expression prevents maternal artery remodeling.

Placentas and fetuses from either LB2V5-CA-HIF-1α (right) or Lv-CMV-GFP-V5 (left) infected blastocysts were collected at embryonic day 14.5 (E14.5) and fixed in paraformaldehyde and embedded in paraffin. Placental sections were hybridized with DIG-labeled riboprobes specific for *Pecam-1* (maternal endothelial cell marker) or *Plf* (specific invasive giant cell marker), denoted by purple staining. DIG-expression was analyzed using alkaline phosphatase immunohistochemistry. Sections were counterstained with nuclear fast red. Arrows indicate tissue surrounding maternal arteries.

Figure 14.



maternal arteries (Figure 14). The presence of maternal endothelial cells, along with the lack of invasive giant cells clearly illustrate that placentas with prolonged HIF-1α expression fail to remodel maternal arteries. Thus, indicating HIF-1α as a major regulator of maternal artery remodeling and placental development.

IV. DISCUSSION

Altered Morphology

Embryonic development occurs under low oxygen levels, and under low oxygen levels of HIF-1α are greater due to an increase in HIF-1α half-life. During implantation, access to oxygen via the maternal blood supply causes a decrease in HIF-1α, due to a rapid proteasomal degradation (half-life of approximately 5 minutes) [60,61]. The access to oxygen resulting in a decrease in HIF-1α expression is believed to be what determines the nature of trophoblast cells in terms of proliferation versus differentiation [24,25]. Therefore, HIF-1α regulation would directly effect placental development.

Our study suggests that prolonged HIF-1α expression dramatically alters placental morphology and possibly function by preventing differentiation into fully functioning trophoblast cells. Trophoblast cells of placentas with prolonged expression of HIF-1α exhibit disorganization with numerous giant cells being expressed in the labyrinthine layer and compaction of the spongiotrophoblast layer. Disorganization was also demonstrated by a decrease in branching morphogenesis of the labrynthine layer, which is responsible for nutrient transport [62]. Although this disorganization did not demonstrate a significant difference in placental or fetal weight at this stage of development (Figure 5), the difference in fetal and placental size may be more pronounced if carried out until birth. One explanation for having no significant difference in fetal weights at E14.5 is that the placental invasion of maternal arteries had just completed, placental invasion is completed near E12 in mice [23]. Placental invasion of maternal arteries is key in placental development and determining whether a pregnancy will be successful; therefore, it may be expected that differences in pup weight

may not be apparent until later in the pregnancy, after the fetus has had time to fully develop [4,9].

Maternal artery remodeling

The results of this study indicated a notable inability of invasive giant cells to remodel maternal arteries. The lack of invasive giant cell function was seen by both the presence of maternal endothelial cells surrounding arteries and a lack of invasive giant cells that should have replaced the maternal endothelial cells through the process of remodeling. Remodeling of maternal arteries is mediated by invasive trophoblast giant cells that destroy maternal endothelial cells and replace them with endovascular trophoblast cells [9]. Pre-eclampsia is a disorder that effects approximately 5-7% of all pregnancies and is believed to result from inappropriate trophoblast invasion and remodeling of maternal arteries, as we have seen in this study [2,7,59,52,63]. However, the exact cause of pre-eclampsia has yet to be elucidated. It is known that pre-eclampsia is characterized by the symptoms of high blood pressure and proteinuria during pregnancy, which can result in early delivery, and possible maternal and/or fetal mortality [6,7,64]. Vascular tension caused by overexpression of HIF-1α in the placenta during pregnancy may be one explanation for the increased blood pressure experienced by those who have pre-eclampsia. It also follows that proteinuria is a result of this increased vascular tension that would also effect the kidneys and their function. Understanding the role of HIF-1α regulation in placental development could aid in our understanding pre-eclampsia as a whole.

Future studies

Although this study provides evidence for the importance of regulation of HIF-1α expression to maintain placental organization and function of certain cell types, further studies must be completed in order to determine the effects of prolonged HIF-1α expression at birth. In order to confirm that the placenta is not plastic in nature, placentas would need to be collected at birth. Additionally, placental and pup weights would be taken at birth to determine if pups are small in comparison to controls because a decrease in birth weight is indicative of pre-eclampsia and other pregnancy associated disorders [6,7,47,64]. Another important aspect of many pregnancy-associated disorders is early delivery. In order to determine if prolonged expression of HIF-1α causes early pup delivery, the time and embryonic day of delivery would need to be recorded. In addition to examining birth weight, placental weight, and time of birth, additional factors that are common in pre-eclampsia could be studied. Since pre-eclampsia is known for a lack of maternal spiral artery remodeling, as was evident in this study at E14.5, the additional symptoms of pre-eclampsia should be analyzed [6,7,9,63,64]. These include high maternal blood pressure, maternal proteinuria, circulating maternal soluble fms-like tyrosine kinase 1 (sFlt1), and glomerular injury [6,7,63-67]. Maternal blood pressure, protein levels, and sFlt1 would need to be tested before embryonic implantation, during pregnancy, and post birth. These points are all necessary because women who experience pre-eclampsia usually exhibit normal blood pressure, low to no urine protein, and normal sFlt1 before or after birth, but display increased blood pressure and sFlt1 levels, as well as proteinuria during pregnancy [6,7,47,64]. Since sFlt-1 is a direct target of HIF-1α, it would be expected that sFlt-1 levels would rise in our mice [63,65].

Maternal kidneys collected at birth could be used to test for glomerular injury. Since there is proteinuria in pre-eclampsia, some glomerular injury would be expected. [6, 65] In a very extreme case of pre-eclampsia known as hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome mothers experience extensive glomerular injury. Checking maternal kidneys would therefore be beneficial in identifying the severity of pre-eclampsia.

Another study that would better elucidate the role of HIF-1α during placental development would include causing increased HIF-1α during specific time-points during placental development. This study could be completed by cloning the oxygen insensitive form of HIF-1α used in this study into a lentiviral, tet-inducible construct. The placenta would be infected in the same manner as in this study; however, the increased expression of HIF-1α would not be induced until addition of doxycycline to the system. By this method of analysis, the critical time-points of placental development governed by HIF-1α could be determined. This method could even allow for increase expression of HIF-1α only during the initial development of the placenta, around E9.5-11.5, followed by normal HIF-1α expression throughout the rest of the pregnancy. The ability to narrow the window of HIF-1α increased expression could greatly increase our understanding of the role of HIF-1α on placental development and pregnancy associated disorders.

V. APPENDIX

Abbreviations

2-ME-2-methoxyoestradiol

ARNT: Aryl hydrocarbon receptor nuclear translocator

CA-HIF: Constitutively active- hypoxia inducible factor

CMV: Cytomegalovirus

COMT: cathechol-O-methyltransferase

DIG-Digoxigenin

d.p.c: days post copulation

E14.5: embryonic day 14.5

FIH: factor inhibiting HIF-1α

Gcm1: glial cell marker 1

GFP: Green Fluorescent Protein

HELLP: hemolysis, elevated liver enzymes, and low platelets

HIF-1α: hypoxia inducible factor 1 alpha

HIF-1β: Hypoxia inducible factor-1β

HRE: Hypoxia responsive element

ICM: inner cell mass

ICR: imprinting control region

IU: international units

KO: Knock out

KSOM: KCL Simplex Optimization Medium

Mash2: Mammalian achaete-scute homologous protein-2

NBT/BCIP: nitro-blue tetrazolium chloride and BCIP 5-bromo-4-chloro-3' indolyphosphate p-toluidine salt

Pecam-1: platelet/endothelial cell adhesion molecule-1

PHD: prolyl hydroxylase domain protein

PBS: phosphate buffered saline

PFA: paraformaldehyde

PL2: Placental lactogen II

Plf: proliferin

PlGF: placental growth factor

pLv: Lentiviral plasmid

PVDF: polyvinylidene fluoride

sFlt1: soluble fms-like tyrosine kinase 1

Tpbpa/ 4311: Trophoblast specific protein alpha

VEGF: vascular endothelial growth factor

VHL: von Hippel-Lindau protein

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