Elucidating the Role of Hypoxia Signaling in Placental Trophoblast Differentiation

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Anatomy and Cell Biology
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Abstract

Preeclampsia is a common and serious complication of pregnancy with no cure except premature delivery. The root cause of preeclampsia is improper development of the placenta, the temporary organ supporting fetal growth. In preeclamptic placentas, a low O$_2$ environment persists due to dysregulated blood flow. My data show that low O$_2$ inhibits differentiation and fusion of progenitor cytotrophoblast cells into a multinucleated syncytiotrophoblast and may thus contribute to the poor placentation in preeclampsia; however, the underlying mechanisms are not well understood. Since low O$_2$ activates a transcription factor complex in cells known as the hypoxia-inducible factor (HIF), I hypothesized that HIF signaling under low O$_2$ impairs placental syncytiotrophoblast formation by altering gene expression. I showed that knockdown of ARNT (a key component of HIF) restores syncytialization under low O$_2$. I also identified downstream targets of HIF that may control this process, which could aid in developing novel therapeutics for preeclampsia.

**Keywords:** placenta, preeclampsia, cytotrophoblast, syncytiotrophoblast, development, differentiation, cell fusion, regulation, hypoxia, hypoxia-inducible factor
Preeclampsia is a common and serious complication of pregnancy and is a leading cause of sickness and death for pregnant women and babies globally. It presents as sudden-onset high blood pressure and blood vessel damage in mothers that can progress to organ failure, stroke, and death of the mother and/or baby. There is no cure except stopping the pregnancy early and delivering the baby, which can result in both immediate and long-term health problems for the prematurely delivered infant. The root cause of preeclampsia is improper development of the placenta – the temporary organ supporting growth of the baby. Developing safe and effective treatments for the underlying placental dysfunction in preeclampsia is challenging since we do not have a complete understanding of why the placenta forms abnormally in these pregnancies. In preeclamptic placentas, a low O₂ environment persists due to dysregulated blood flow. My data show that low O₂ inhibits placental stem cell differentiation and may thus contribute to the poor placental development/function characteristic of pregnancies with preeclampsia; however, why this happens is not well understood. I suspected that the hypoxia-inducible factor (HIF), a molecule activated within cells under low O₂ to help cells adapt, plays an important role in the inhibition of placental stem cell differentiation and in the development of preeclampsia. The goal of this study was to assess whether HIF signaling under low O₂ inhibits placental stem cell differentiation, and if so, to identify genes regulated by HIF that underlie this inhibition and could be targeted to restore differentiation. My results demonstrated that blocking HIF signaling restores the capacity of placental stem cells to differentiate when cultured under low O₂, suggesting that HIF contributes to poor stem cell differentiation in low O₂ environments. I also identified downstream targets of HIF that may be responsible for inhibiting placental stem cell differentiation under low O₂ conditions. Next steps include determining whether altering expression of certain HIF target genes could alleviate impaired differentiation in low O₂. This study will open doors to develop and test novel therapeutics that could restore placental stem cell differentiation and improve prevention and management of preeclampsia.
Co-Authorship Statement

All data presented in this thesis were collected and analyzed by Adam Jaremek.

Mariyan J. Jeyarajah, Gargi Jaju Bhattad, and Dr. Stephen J. Renaud contributed to the approach and design of experiments.

Dr. Stephen J. Renaud and Dr. Patrick Lajoie assisted in editing this thesis.
Acknowledgements

First and foremost, I would like to thank my incredible supervisor, Dr. Stephen J. Renaud. I am forever grateful for the opportunity to be a part of your lab team and for all your excellent mentorship, teaching, and encouragement throughout the years. Your immense knowledge, creativity, and passion for scientific discovery and improving public health are inspiring and have guided me immensely. Thank you sincerely for all your leadership and for creating an inclusive and engaging lab environment that has played a major part in both my professional and personal growth.

I also express my deepest gratitude to the members of my advisory committee, Dr. Andrew Watson, Dr. Martin Duennwald, and Dr. Patrick Lajoie, for all their extensive guidance, kind support, and constructive feedback in this project. Your vast expertise and insightful perspectives have been so valuable, and it has truly been a pleasure to work with you all.

To current and previous members of the Renaud lab, thank you for all your help, direction, and friendship. Thank you for the many stimulating discussions, for teaching me different techniques, for sharing your skills, advice, and opinions, and for all your collaboration and compassion both inside and outside the lab. Special thanks to both Mariyan J. Jeyarajah and Gargi Jaju Bhattad for being key sources of guidance and for always taking the time to answer my questions or offer me a helping hand. I am so grateful to have been able to work with such friendly, enthusiastic, and thoughtful scientists over the years.

Last but not least, I would like to thank my family and close friends for their unconditional support throughout my studies and for motivating me to keep on learning and improving every day. This thesis is not only the outcome of years of work, but also the people who influenced my life choices that led me to this point. Thank you all for believing in me.
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<th>Description</th>
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<tbody>
<tr>
<td>CTB</td>
<td>Cytotrophoblast</td>
</tr>
<tr>
<td>STB</td>
<td>Syncytiotrophoblast</td>
</tr>
<tr>
<td>EVT</td>
<td>Extravillous cytotrophoblast</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens-1</td>
</tr>
<tr>
<td>ERV</td>
<td>Endogenous retroviral</td>
</tr>
<tr>
<td>STBEV</td>
<td>Syncytiotrophoblast extracellular vesicle</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>PGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>TS</td>
<td>Trophoblast stem</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cell</td>
</tr>
<tr>
<td>hPSC</td>
<td>Human pluripotent stem cell</td>
</tr>
<tr>
<td>PE</td>
<td>Preeclampsia</td>
</tr>
<tr>
<td>FGR</td>
<td>Fetal growth restriction</td>
</tr>
<tr>
<td>iCTB</td>
<td>Interstitial cytotrophoblast</td>
</tr>
<tr>
<td>eCTB</td>
<td>Endovascular cytotrophoblast</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>EPAS1</td>
<td>Endothelial PAS domain 1</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td><strong>EP300</strong></td>
<td>E1A binding protein p300</td>
</tr>
<tr>
<td><strong>HRE</strong></td>
<td>Hypoxia response element</td>
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<tr>
<td><strong>FIH-1</strong></td>
<td>Factor inhibiting HIF-1</td>
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<tr>
<td><strong>CITED2</strong></td>
<td>CBP/EP300-interacting transactivator-2</td>
</tr>
<tr>
<td><strong>FBS</strong></td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td><strong>BSA</strong></td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td><strong>HEK</strong></td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td><strong>DAPI</strong></td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td><strong>qRT-PCR</strong></td>
<td>Quantitative RT-PCR</td>
</tr>
<tr>
<td><strong>SDS</strong></td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td><strong>PI</strong></td>
<td>Propidium iodide</td>
</tr>
<tr>
<td><strong>shRNA</strong></td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td><strong>GFP</strong></td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td><strong>EdU</strong></td>
<td>5-ethyl-2′-deoxyuridine</td>
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<tr>
<td><strong>ChIP</strong></td>
<td>Chromatin immunoprecipitation</td>
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<tr>
<td><strong>ChIP-seq</strong></td>
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<tr>
<td><strong>GO</strong></td>
<td>Gene Ontology</td>
</tr>
<tr>
<td><strong>KEGG</strong></td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
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<tr>
<td><strong>SUPYN</strong></td>
<td>Suppressyn</td>
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Chapter 1 is adapted from Jaremek, A. et al. (2021). Omics approaches to study formation and function of human placental syncytiotrophoblast. Frontiers in Cell and Developmental Biology, 9:674162. Text and figures were reproduced for this thesis with permission from Frontiers. Adam Jaremek, Mariyan J. Jeyarajah, and Stephen J. Renaud wrote and edited the manuscript. Gargi Jaju Bhattad edited the manuscript and contributed to preparation of the tables.

Chapter 2 is adapted from Jaremek, A. & Renaud S. J. (2022). Analyzing trophoblast fusion using immunofluorescence and split protein complementation assays. Accepted and soon to be published; Trophoblast: Methods and Protocols, Methods in Molecular Biology, Humana Press. Text and figures were reproduced for this thesis with permission from Springer Nature. Adam Jaremek and Stephen J. Renaud wrote and edited the manuscript.
Chapter 1: Introduction

1.1. The Human Placenta

The placenta is a temporary organ that forms during pregnancy. It connects the fetus to the uterine wall and serves crucial functions to sustain pregnancy, promote fetal growth and development, and stimulate adaptive changes in maternal physiology and metabolism. These functions include (but are not limited to) hormone production, hemodynamic adaptations, and serving as a barrier between the mother and fetus\(^1,2\). Placental secretions and debris enter the maternal and fetal circulations to modulate maternal physiology, including changes in the cardiovascular, pulmonary, immune, and metabolic systems, as well as regulate fetal growth, parturition, and other functions. For instance, hormones released by the placenta stimulate mammary gland development for subsequent lactation or suppress the maternal immune system from mounting a response against the conceptus\(^3\). In addition, the placenta metabolizes various substances including hormones and releases metabolites into maternal and/or fetal circulations to support the pregnancy. As a physical barrier, the placenta enables transport of vital nutrients, such as \(O_2\), water, carbohydrates, amino acids, and lipids to the developing fetus, while facilitating removal of \(CO_2\) and other waste products from fetal to maternal blood. As an immunological barrier, the placenta also protects the fetus from potentially harmful factors, such as toxins, pathogens, and maternal immune reactivity\(^1,3\).

1.2. Structure and Formation of the Placenta

After fertilization of the oocyte in the ampullary region of the fallopian tube, the resulting zygote undergoes multiple rounds of cell division and differentiates into a blastocyst as it travels down the oviduct and enters the uterus around day 5 post-fertilization. At this stage, cells in the blastocyst are arranged in two layers: an inner cell mass, which goes on to form the embryo, yolk sac, and other structures, and an outer layer of epithelial trophoblast cells called trophectoderm, which contributes to the placenta and nourishes the embryo as it
develops. Approximately 7-9 days post-fertilization, the embryonic pole of the blastocyst becomes attached to the uterine surface epithelium through interactions between adhesion molecules expressed on both trophoblast cells and uterine epithelium. By the time the blastocyst penetrates between the luminal epithelial cells at the point of attachment, trophoblast cells at the embryonic pole have differentiated into an inner layer of mononucleated progenitor cytotrophoblast (CTB) and an outer layer of multinucleated primitive syncytiotrophoblast (STB), that likely forms through intercellular fusion of the underlying CTBs. The primitive STB facilitates implantation of the blastocyst by rapidly expanding into the maternal decidua and eroding uterine stroma, glands and capillaries. The cavities generated within the primitive STB, called lacunae, become filled with blood and glandular secretions from eroded decidual tissue, providing a source of early nutrition for the conceptus.

Following complete penetration of the embryo around day 10 post-fertilization, early formation of the chorionic villi, the functional units of the placenta, begins. At about days 13 and 14, rows of proliferative CTBs break through the expanding primitive STB and form primary villi. These primary villi traverse the entirety of the primitive STB and branch extensively to create smaller floating villi within the spaces between villi (called intervillous spaces) to increase the surface area of the villous tree. Anchoring villi are formed when proliferative, non-polarized CTBs break through the overlying syncytiotrophoblast and form columns of cells that invade into the uterine wall, attach to the decidua basalis, and connect laterally with similar extensions of neighboring villi to encircle and anchor the conceptus as the CTB shell. A subpopulation of CTBs in the proximal part of these cell columns eventually give rise to invasive and migratory extravillous cytotrophoblast (EVT) cells that continue to invade into the decidua and inner third of the myometrium. To support fetal development, these cells evoke profound changes within the uterine spiral arteries that course through the decidua basalis, which ultimately allows for sufficient maternal blood flow to the intervillous space.

During the third week of development, chorionic villi are formed when extraembryonic mesoderm and blood vessels emanating from the allantois infiltrate the proximal cores of the primary villi. Thus, the villus core includes an inner meshwork of mesoderm-derived
stroma consisting of fibroblasts and immune cells (notably macrophages) as well as blood vessels contiguous with the fetal circulation via the umbilical vessels. The core is lined by a trophoblast bilayer consisting of an outer STB layer, known as the definitive STB at this stage, and an inner CTB layer, which are both physically separated from the stroma by a laminin-rich basement membrane. The definitive STB forms by fusion and differentiation of the underlying villous CTBs, but whether it represents a distinct entity or the gradual evolution of the primitive STB as gestation progresses remains unclear. Since STB lines the outer surface of the villi, it directly bathes in maternal blood and forms a key site of interaction between maternal and fetal tissue. The STB plays crucial roles in controlling exchange of nutrients, wastes, and gases between maternal and fetal blood, and possesses endocrine activity, releasing key hormones involved in the homeostasis of pregnancy. STB secretions and debris are also deposited into maternal circulation, where they have vital roles in modulating maternal physiology as well as diagnostic potential for fetal-placental aberrations and pregnancy disease\textsuperscript{7,11}. Despite the importance of STB for fetal development and pregnancy outcome, the mechanisms underlying its formation and function are still relatively understudied. Hence, better understanding STB development is a key goal of this thesis. A schematic illustrating the primitive and definitive STB is presented in Figure 1.1.
Figure 1.1. Sequential development of STB.

(A) Progression of a human embryo at about gestational days 7–8 (peri-implantation; left image) and days 9–10 (post-implantation; right image). Please note the invasive properties of the primitive STB at the forefront of the implanting human embryo and the gradual development of blood-filled lacunae. (B) Cross section of a chorionic villus in later gestation. Please note that the STB layer exhibits apical-basal polarity (as shown by the presence of microvilli) and bathes directly in maternal blood. An extruding syncytial knot is also shown. The villus core contains blood vessels that connect to the fetal circulation as well as different cell types (such as Hofbauer cells and fibroblasts). CTBs are shown in various stages of their life cycle (proliferating, differentiating, and fusing).
1.3. Differentiation of CTB into STB

STB has a limited lifespan and must be regularly replenished throughout pregnancy with fresh cytoplasm and nuclei by controlled differentiation and fusion of underlying CTBs. Differentiation and fusion of CTBs into STB is a complex and highly orchestrated process that involves biochemical changes to support the immense endocrinological and secretory functions of STB, as well as morphological changes to enable intercellular fusion. It is not yet clear whether the signal that initiates these biochemical and morphological events originates from the STB or CTB layer. Additionally, while biochemical and morphological differentiation are coupled, they are thought to be executed by discrete pathways. Biochemical differentiation requires that CTBs exit the cell cycle and repress genes involved in maintaining a progenitor state, such as ELF5, TP63, ID2, and TEAD4. At the same time, factors implicated in nutrient transport, immunomodulation, and hormone biosynthesis and metabolism are induced. The integration of multiple signaling pathways and transcription factors is implicated in the process of CTB differentiation, including suppression of WNT and activin/transforming growth factor beta (TGF-β) signaling as well as activation of the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) pathways. CTB differentiation also involves the activity of many transcription factors and epigenetic regulators including PPARG, DLX3, GCM1, TFAP2A, OVOL1, and many others.

Morphologically, CTB fusion necessitates a modified epithelial-mesenchymal transition resulting in the loss of junctional proteins such as E-cadherin or zonula occludens-1 (ZO-1), reorganization of cytoskeletal components, and intercellular mixing of intracellular contents. The impetus for intercellular fusion is largely mediated by expression of cellular fusogens called syncytins. Syncytins are encoded by co-opted endogenous retroviral (ERV) envelope genes ERVW-I (encodes syncytin-1) and ERVFRD-1 (encodes syncytin-2). Syncytin-1, which is expressed in STB, binds to the neutral amino acid transporter ASCT2 expressed mainly by CTBs. Syncytin-2, on the other hand, is expressed in small clusters of CTBs and binds to MFSD2A, which is expressed by STB. Additionally, changes in the cytoskeleton are required to form the extensive microvilli that cover the apical surface of STB and increase the surface area of the STB up to seven-fold.
1.4. STB Life Cycle

STB undergoes highly regulated turnover as aged or damaged syncytia are replaced by newly formed ones through fusion of underlying CTBs\textsuperscript{20}. Since this occurs continuously from implantation until term, the nuclei present in STB are of different ages and exhibit a range of morphologies and packing densities that reflect progressive maturation. Within STB, clustering of nuclei occurs in regions known as syncytial sprouts and knots\textsuperscript{21}. Syncytial sprouts, which are predominant during the first-trimester, harbor nuclei that are primarily euchromatic with a distinct nucleolus. They form protrusions in the development of new villi, yet their connection with the villus surface can become attenuated and render them susceptible to detachment and release into the intervillous space\textsuperscript{22}. Syncytial knots, which often protrude from the surface of villi during the third trimester, contain more densely clustered nuclei that may be less transcriptionally active based on features such as dense condensations of heterochromatin and lack of apparent nucleoli\textsuperscript{23}. Although the nuclei resemble those classified as apoptotic, whether syncytial knots represent an apoptotic end-stage of the STB life cycle remains elusive as nuclear fragmentation is not observed\textsuperscript{21}. Nevertheless, knots are considered a means by which aged STB nuclei are sequestered to regions of the villus membrane where they do not interfere with exchange\textsuperscript{24}, and some normally detach to be shed into maternal circulation\textsuperscript{25}. The volume of syncytial knots relative to CTB volume increases during gestation, suggesting that early proliferation is geared towards growth with later proliferation towards renewal and repair\textsuperscript{26}.

Over the course of pregnancy, STB releases a variety of factors into maternal circulation that are critical for the maintenance of healthy pregnancy. This includes fragments derived from syncytial sprouts or knots, which range from small subcellular particles to large multinucleated fragments, that may play important roles in maintaining maternal immune tolerance to fetal tissues\textsuperscript{27}. Furthermore, STB releases membrane-bound vesicles known as STB extracellular vesicles (STBEVs) in the form of exosomes, microvesicles, or apoptotic bodies, from the villus surface into maternal circulation\textsuperscript{28}. These vesicles contain a variety of biologically active molecules, such as proteins, RNAs, and lipids, that have regulatory roles in the maternal immune response to pregnancy and may interact with components of maternal circulation, such as endothelial cells or leukocytes, to facilitate maternal-fetal
communication\textsuperscript{29}. STB also releases cell-free ‘fetal’ DNA into maternal blood that varies in concentration based on multiple factors including oxidative stress\textsuperscript{30}. Additional factors produced and released by STB include numerous steroid and peptide hormones, such as estrogen, progesterone, and human chorionic gonadotropin (hCG), as well as hormone-metabolizing enzymes, such as hydroxysteroids\textsuperscript{31}. For instance, transition of CTBs to endocrinologically competent STB involves induction of genes, such as \textit{CGA} and \textit{CGB} encoding the $\alpha$ and $\beta$ subunits of hCG, respectively, as well as \textit{HSD11B2}, encoding 11$\beta$-hydroxysteroid dehydrogenase, which converts active cortisol into inactive cortisone\textsuperscript{32}. STB also produces a variety of growth factors, such as pregnancy-specific glycoproteins, vascular endothelial growth factor, placental growth factor (PGF), TGF-$\beta$, and many other cytokines, chemokines, and signaling molecules\textsuperscript{33}.

1.5. Models to Study STB Development

Since the placenta is expelled at the end of pregnancy (i.e., early pregnancy termination or delivery) and is often considered clinical waste, it is possible to conduct experiments using placental tissue. Moreover, unlike many other tissues used for \textit{ex vivo} analyses that are biopsied or removed only when diseased, it is possible to collect placental tissue from pregnancies deemed healthy. To study STB biology, villus explants can be cultured for defined time periods, which is advantageous to study STB function while preserving tissue integrity\textsuperscript{34}. CTBs can also be isolated and enriched from placental tissue\textsuperscript{35}. Isolated CTBs spontaneously differentiate into STB following removal from intact tissue and are considered a reliable representation of STB generation. However, since isolated CTBs have a limited capacity for proliferation in culture, they are not well suited for mechanistically studying repression of proliferation during early stages of STB formation. Therefore, choriocarcinoma cell-lines are also valuable for the study of STB biology, due to their resiliency and extended lifespans in culture. BeWo cells were derived from a brain metastasis, serially cultivated, and are adapted to cell culture\textsuperscript{36}. Differentiation of BeWo cells into hormone-producing STB-like cells is stimulated following exposure to agents such as forskolin\textsuperscript{37}. Forskolin activates adenylate cyclase, which increases intracellular levels of cAMP, thereby stimulating cAMP-sensitive pathways implicated in STB generation such as the PKA pathway\textsuperscript{38}. Other commonly used choriocarcinoma cell-lines,
including JEG-3, JAR, and ACH3P, produce hCG in response to forskolin, but do not fuse under standard culture conditions\textsuperscript{39,40}. Thus, their utility as models of STB development is limited.

In 2018, culture conditions to maintain trophoblast stem (TS) cells from human embryos or first-trimester placentas were determined\textsuperscript{41}. These cells can be maintained as CTB-like cells or stimulated to form STB-like or EVT-like cells by altering media components. Organoid cultures of human trophoblasts derived from first-trimester placenta have also been established, which provide a powerful model to study human STB biology while considering the three-dimensional (3D) spatial configuration\textsuperscript{42,43}. Human embryonic stem cells (hESCs) and pluripotent stem cells (hPSCs) cultured under defined culture conditions can also differentiate into cells with features consistent with trophoblasts, including STB\textsuperscript{44–46}. In particular, recent reports indicate that naïve hPSCs can be used to model the entire trophoblast lineage trajectory from trophectoderm through CTBs to STB\textsuperscript{47–49}. STB derived from hESCs and hPSCs also offer the possibility of studying normal and pathological STB development from distinct genetic backgrounds\textsuperscript{50}. The cell models commonly used to study human STB are summarized in Table 1.1. It is noteworthy that animal models with a syncytialized placental barrier (including rodents and primates) have been instrumental in providing insight into STB formation and function. In many cases, factors identified as critical for STB formation in animal models have subsequently been shown to have a conserved function in human STB development\textsuperscript{7}.
Table 1.1. Cell models used to study STB development and function

<table>
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<tr>
<th>Source</th>
<th>Model</th>
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<th>Notes</th>
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<tbody>
<tr>
<td>Placenta</td>
<td>Placental Villus Explants</td>
<td>34,51</td>
<td>Prepared by dissecting placental tissue and incubating in tissue culture wells for defined time periods. Denudation and regeneration of STB is also possible. Benefits include preservation of tissue integrity. Challenges include minimizing variation within and between experiments due to heterogeneity of explant preparation.</td>
</tr>
<tr>
<td>Placental (CTB) Organoids</td>
<td></td>
<td>42,43</td>
<td>Derived from early gestation placentas and can be expanded and cultured long-term. Provides a powerful model to study STB formation in 3D. Of note, the STB layer faces toward the inside of the organoid, so modelling transplacental passage of substrates may be limited.</td>
</tr>
<tr>
<td>Human TS Cells</td>
<td></td>
<td>41</td>
<td>Derived from early gestation placentas or human blastocysts. Can be maintained as CTB-like cells in the presence of GSK-3, TGF-β, and HDAC inhibitors. Cells form STB-like cells after removing these inhibitors and adding forskolin.</td>
</tr>
<tr>
<td>Primary CTBs</td>
<td></td>
<td>35,52</td>
<td>Isolated and enriched from placentas following delivery. Advantageous as they have undergone few population doublings or manipulations, and can spontaneously form STB in culture. Cells have limited capacity to proliferate in culture, so they are less well suited to study early stages of syncytialization. Contamination with unwanted cell types and changes in CTB viability after isolation can pose a challenge.</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>BeWo</td>
<td>36,53</td>
<td>Cells have extended lifespans in culture. Beneficial for studying molecular aspects of cell fusion and hormone production, but possess genetic signatures distinct from normal trophoblast, so results should be interpreted with caution. Exposing BeWo cells to cAMP agonists stimulates STB-like cell fusion and hormone production. JEG-3, JAR, and ACH3P produce hormones (hCG) in response to cAMP agonists, but do not fuse, so their utility for modeling STB formation is limited.</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>JEG-3</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>JAR</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>ACH3P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early-stage embryos</td>
<td>hESCs</td>
<td>44,45</td>
<td>Using defined culture conditions, hESCs and hPSCs differentiate into cells possessing trophoblast-like properties, including STB-like cells. Beneficial for studying normal and pathological STB development from distinct genetic backgrounds, although there is contention about whether these cells truly represent trophoblast. Cells derived from naïve hESCs and hPSCs (rather than primed hESCs and hPSCs) appear to form bona fide trophoblast and can delineate the entire trophoblast developmental trajectory from pre- to post-implantation, including STB formation.</td>
</tr>
<tr>
<td>Reprogrammed somatic cells</td>
<td>Primed hPSCs</td>
<td>50,56,57</td>
<td></td>
</tr>
<tr>
<td>Reprogrammed somatic cells</td>
<td>Naïve hPSCs</td>
<td>47-49</td>
<td></td>
</tr>
</tbody>
</table>
1.6. STB in Pregnancy Disease

Hypertensive disorders of pregnancy are a leading cause of severe morbidity and mortality among mothers and infants. Preeclampsia (PE) is a pathology that falls within the spectrum of hypertensive disorders of pregnancy and affects 5-8% of all pregnant women. This amounts to 20,000 cases annually in Canada as well as approximately 76,000 maternal deaths and 500,000 fetal and newborn deaths globally each year\textsuperscript{58,59}. There are two major sub-types of PE classified based on timing of symptom onset: early and late-onset PE, with a start of clinical signs and symptoms before and after 34 weeks, respectively\textsuperscript{60}. PE is clinically defined as the presence of new-onset maternal hypertension (blood pressure $\geq 140/90$ mmHg) and proteinuria ($\geq 300$ mg/day) during the second half of pregnancy. Severe PE includes even greater hypertension ($\geq 170/110$ mmHg), heavy proteinuria, as well as additional signs of potential maternal end-organ dysfunction, including hemolysis, elevated liver enzymes (impaired liver function), and thrombocytopenia (low platelets)\textsuperscript{61,62}. Other complications may include pulmonary edema, new-onset renal insufficiency, and visual disturbances\textsuperscript{63}. Post-delivery, PE is associated with an increased risk of developing chronic hypertension, ischaemic heart disease, venous thromboembolism, heart failure, and stroke, as well as a decreased life expectancy\textsuperscript{64–67}. There is currently no definitive cure for PE except removal of the placenta and thus the early termination of pregnancy\textsuperscript{68}.

PE is also a major cause of serious fetal and neonatal complications. For instance, prematurity and low birth weight due to preterm delivery (delivery prior to 37 weeks of pregnancy) can increase immediate risk of perinatal death as well as predispose the prematurely delivered fetus to serious chronic diseases later in life, such as diabetes, cardiovascular, and renal diseases\textsuperscript{59,69}. Furthermore, PE is associated with fetal growth restriction (FGR), which can occur with or without PE; approximately 30% of PE cases are complicated by FGR\textsuperscript{70}. This condition affects 3-9% of all pregnancies and occurs when the fetus is smaller than expected for the gestational age and is unable to reach its growth potential as determined by genetic and epigenetic factors\textsuperscript{71,72}. Clinically, it is often defined as an estimated fetal weight <10\textsuperscript{th} percentile due to a pathological process, and it is linked to poor health outcomes. This includes increased risk of perinatal mortality and morbidity,
including neonatal respiratory distress syndrome, infections, poor neurodevelopmental outcomes, cognitive impairments, hypertension, obesity, and type II diabetes\textsuperscript{71,73–75}.

Central to the etiology of pregnancy complications such as PE and FGR is abnormal development and function of the placenta. Placentas of pregnancies complicated by FGR are characterized by impaired vasculogenesis and angiogenesis, poor villous growth, as well as a smaller surface area of STB, which can have adverse consequences including insufficient transport of nutrients and gases between mother and fetus as well as poor fetal protection against maternal immune responses\textsuperscript{59,76,77}. Furthermore, a key hallmark of PE is the abnormal differentiation of placental trophoblasts, including deficient STB formation. For example, PE-related histological changes in the placenta include a decreased number of syncytial microvilli, smaller and atrophic villi, and dilated spaces separating a thinner, degenerated STB from the underlying structures\textsuperscript{59,78}. Cultured CTBs from PE or FGR-affected placentas also show impaired cell fusion and reduced expression of critical fusion mediators\textsuperscript{79}, and in STB from these placentas, there is a greater number of apoptotic nuclei present\textsuperscript{80}. PE is associated with increased syncytial knotting as well as greater extrusion of STB fragments and pro-inflammatory STBEVs implicated in immune dysregulation and endothelial damage\textsuperscript{29,59}. In addition, there is an altered composition of placental proteins within STBEVs isolated from plasma of women with pregnancy-related disorders such as PE, which holds promise to be exploited as potential biomarkers for early diagnosis and monitoring\textsuperscript{81}. Changes in STB function during disease states are summarized in Figure 1.2.
Figure 1.2. Schematic illustrating the multifunctional importance of STB for a healthy pregnancy.

A cross section of a placental chorionic villus near term is shown. O₂, micronutrients, immunoglobulins, water, and various other substances pass across the STB layer to gain access to blood vessels in the villus core, through which they can be carried to the fetus. CO₂ and waste products diffuse across the STB layer from fetal to maternal blood. STB also produces and secretes a variety of factors into maternal blood, including peptide and steroid hormones, growth factors, STBEVs, and larger vesicles (e.g., syncytial knots). Given the diverse functions of STB for pregnancy success and its contiguity with maternal blood, aberrant STB function can contribute to adverse pregnancy outcomes.
1.7. O₂ Regulation in Placental Development and Disease

Throughout gestation, there are drastic alterations in O₂ tensions within the placenta that accompany and guide normal embryogenesis and placentation. In placental anchoring villi, a subset of proliferative CTBs that form cell columns gain migratory capabilities and upon detachment, begin to differentiate into two types of early EVTs: interstitial CTBs (iCTBs) that migrate into the decidual stroma, and endovascular CTBs (eCTBs) that migrate into the maternal spiral arteries. During the first 10-12 weeks of gestation, the placenta develops under a low O₂ environment of about 1-3% O₂, which is attributable to a lack of significant blood flow to the intervillous space as a result of eCTBs invading and plugging the distal portions of uterine spiral arteries. This relatively low O₂ environment is thought to limit oxidative stress to the placenta and developing fetus as well as promote CTB proliferation and differentiation into immature EVTs. At approximately 10-12 weeks of gestation, the trophoblast plugs are gradually displaced, and blood flow is increased to fulfill the demands of the growing fetus. Consequently, the O₂ levels within the intervillous space rise (eventually to about 8% O₂), and an increased O₂ exposure is thought to facilitate CTB (particularly eCTB) differentiation into fully mature EVTs. The mature EVTs displace the endothelium and smooth muscle of the spiral arteries to transform them into low resistance, high capacitance vessels. This in turn increases blood vessel compliance and reduces vasoreactivity and pulsatility to allow a steady stream of blood into the placenta and ensure adequate opportunity for maternal-fetal exchange.

While a low O₂ environment during the first trimester is physiological, a failure in the O₂ transition to occur properly can lead to pregnancy complications such as PE. Shallow EVT invasion and poor uterine spiral artery remodeling can result in impaired/intermittent blood flow as well as persistent placental hypoxia and oxidative stress, and it is strongly linked to the development of PE and FGR. A persistently low O₂ tension after 12 weeks of gestation likely contributes to the impaired STB formation that is characteristic of PE or FGR-affected placentas. For example, CTBs isolated from placentas of various gestational ages show impaired cell fusion and secretion of STB-specific proteins, such as hCG and PGF, when cultured under low O₂ levels. Also, compared to standard culture conditions, multiple cell-lines including primary-term CTBs and BeWo cells cultured under low O₂
show reduced levels of GCM1, a transcription factor that controls expression of the cellular fusogens syncytin-1 and syncytin-2, which could explain the deficient cell fusion observed\textsuperscript{93–98}. Correspondingly, PE placentas also show decreased GCM1, syncytin-1, and syncytin-2 levels as well as impaired syncytialization\textsuperscript{59,99,100}. Several other studies have attempted to uncover the mechanisms by which low O\textsubscript{2} represses STB development. For instance, induction of the microRNA miR-210 under low O\textsubscript{2} can inhibit STB formation in BeWo cells\textsuperscript{101}. The transcription factor ASCL2 is also upregulated under low O\textsubscript{2} in primary CTBs and is thought to inhibit syncytialization through induction of downstream transcription factors USF1 and USF2, which repress important STB marker genes\textsuperscript{102,103}. Epigenetic modifications also likely play a role; culture of primary CTBs under low O\textsubscript{2} conditions stimulates hypermethylation at specific genomic sites that potentially decreases expression of genes crucial for STB formation\textsuperscript{104}. Elucidating how placental hypoxia can inhibit CTB differentiation and fusion into STB is of critical importance to better understand the pathogenesis of pregnancy complications such as PE and FGR. Thus, in this thesis, I investigate how activation of a particular highly conserved transcriptional complex under low O\textsubscript{2} conditions may contribute to impaired STB development.

1.8. Hypoxia Inducible Factor

Low O\textsubscript{2} serves as a cellular trigger to alter metabolism and activate adaptive molecular responses, such as promoting delivery of O\textsubscript{2}. In mammalian cells, the main regulator of the cellular response to hypoxia is a transcription factor complex known as the hypoxia-inducible factor (HIF). HIF is a member of the basic helix-loop-helix transcription factor family and is a heterodimer composed of a variable and regulatory α subunit that is sensitive to O\textsubscript{2}, and a constitutively expressed β subunit insensitive to O\textsubscript{2} known as HIF1β, also referred to as aryl hydrocarbon receptor nuclear translocator (ARNT)\textsuperscript{105,106}. The mammalian genome encodes three isoforms of the α subunit, including HIF1α, HIF2α (also referred to as endothelial PAS domain 1 or EPAS1), and HIF3α. HIF1α is the most extensively studied and has the broadest tissue distribution, suggesting roles in homeostasis, while HIF2α and HIF3α are expressed in a more restricted pattern, suggesting they have more specialized and tissue-specific regulatory functions\textsuperscript{107}. Under O\textsubscript{2} replete conditions, the HIF α subunits become hydroxylated at conserved prolyl residues by a
family of O₂ sensitive prolyl hydroxylase domain enzymes (PHD1-3). This generates a binding site on the HIF α subunits for the von Hippel-Lindau (VHL) tumor suppressor protein, which ubiquitinates and targets the subunits for rapid proteosome-mediated degradation. However, under low O₂ conditions, the α subunits are no longer hydroxylated and are stabilized, allowing them to translocate into the nucleus, dimerize with ARNT, recruit coactivators including CREB binding protein (CBP) and E1A binding protein p300 (EP300), and subsequently bind to hypoxia response elements (HREs) in the DNA containing the core consensus sequence 5’-RCGTG-3’. This in turn regulates transcription of the associated hypoxia-responsive target genes, which are involved in erythropoiesis, angiogenesis, proliferation, differentiation, and/or metabolism. A simplified schematic of HIF signaling regulation in O₂ replete and deficient conditions is presented in Figure 1.3.

In addition to protein stability, the transcriptional activity of HIF can be modulated by other regulatory factors/proteins. Factor inhibiting HIF-1 (FIH-1) is an O₂ sensitive asparaginyl hydroxylase that hydroxylates a conserved asparagine residue on the HIF α subunits under sufficient O₂ and prevents their interaction with the transcriptional coactivators EP300 and CBP. CBP/EP300-interacting transactivator-2 (CITED2) also functions as a negative feedback regulator of HIF1α; it is induced under low O₂ and outcompetes HIF1α for EP300/CBP binding. Furthermore, HIFs may respond differentially depending on the severity and length of low O₂ exposure due to their unique regulation. For example, it is proposed that HIF1α stabilization under hypoxia is an acute response that is diminished under prolonged periods of low O₂, while HIF2α levels continue to increase with time, suggesting that HIF2α-mediated gene transcription is more important under conditions of chronic low O₂ exposure. The role of HIF3α under low O₂ conditions is less well known. Also, it is important to note that components of the HIF pathway can be regulated by factors independent of O₂ levels. For instance, PHDs can be negatively regulated by nitric oxide, metabolites, or reactive oxygen species, while HIF1α levels can be induced by hormones, cytokines, and growth factors.
Figure 1.3. Schematic illustrating the O$_2$-dependent regulation of HIF signaling.

Under O$_2$ replete conditions (left panel), PHDs hydroxylate HIF α subunits (e.g., HIF1α) on specific prolyl residues. This allows VHL to bind and ubiquitinate HIF1α, resulting in rapid proteasome-mediated degradation. Under low O$_2$ conditions (right panel), inhibition of PHD activity stabilizes HIF1α and allows it to translocate into the nucleus, dimerize with HIF1β/ARNT, recruit CBP/EP300, and bind to HREs in the DNA to then regulate transcription of the associated hypoxia-responsive genes.
1.9. HIFs in Placental Development and Disease

Activation of the HIF signaling pathway in response to low O$_2$ is strongly implicated as a critical regulator of placental development. For instance, studies using transgenic mouse models have shown that components of HIF contribute in fundamental ways. Specifically, mice possessing null mutations of ARNT, or both HIF1α and HIF2α, show severe defects in placentation including shallow placental invasion, impaired vascularization, and altered trophoblast tissue formation (including deficient STB generation) leading to embryonic lethality at mid-gestation$^{112,113}$. Disruption of HIF1α or HIF2α alone similarly leads to mid-gestation embryonic death, but with placental phenotypes that are not as severe as ARNT or HIF1α and HIF2α double nulls, suggesting some redundancy between HIF1α and HIF2α functions during placentation$^{112,114,115}$. Responses to changing placental O$_2$ tensions driven by HIF signaling are also proposed to guide trophoblast lineage decisions and affect how the placenta forms a functional interface with the maternal environment. This can be illustrated by the sensitivity of the invasive EVT lineage to changes in O$_2$ levels$^{85}$. For instance, culture of first-trimester primary CTBs or hPSC-derived CTBs under low O$_2$ inhibits CTB fusion and expression of STB markers, while directing differentiation towards the EVT lineage, as indicated by an increase in EVT-specific markers such as HLA-G and ASCL2. This effect was dependent on an intact HIF complex, since knockdown of HIF1β/ARNT abrogated the phenotype$^{50,89}$.

During early pregnancy, HIF1α and HIF2α proteins are highly expressed in STB, CTBs, and fetoplacental vascular endothelium, and their expression is reduced after the first trimester concomitant with the initiation of maternal blood flow to the intervillous space and the resulting increase in physiological O$_2$ tension$^{116,117}$. However, in placentas from pregnancies complicated by PE or FGR, expression of HIF1α and HIF2α remain elevated when compared to healthy term placentas. This is likely due to a combination of insufficient remodeling of maternal spiral arteries, resulting in poor blood flow and thus persistent placental hypoxia, as well as the impaired O$_2$-dependent, proteosome mediated degradation of HIF1α and HIF2α due to deficient interactions with VHL and proteasome dysfunction$^{118-121}$. Recently, Colson et al. showed that the inhibition of STB formation under low O$_2$ in primary term CTBs could be partially prevented through knockdown of
HIF2α, suggesting that HIF2α transcriptional activity plays a role\textsuperscript{122}. Collectively, these findings suggest that activation of hypoxia/HIF signaling in PE or FGR-affected placentas likely contributes to the impaired CTB fusion and differentiation into STB.

1.10. Rationale

Placental O\textsubscript{2} tension plays an important role in regulating trophoblast differentiation and function, both in normal and pathological pregnancies. For instance, STB development is influenced by low O\textsubscript{2}, but the underlying mechanisms have yet to be fully elucidated. HIF is a heterodimeric transcription factor that is activated in cells under low O\textsubscript{2} to help cells adapt and is suggested to play a role in the control of CTB differentiation and fusion into STB. However, the direct downstream targets of HIF and molecular pathways involved are currently not well known, so addressing this gap in knowledge is a major aim of this thesis. A comprehensive understanding of HIF transcriptional activity would unveil the detailed, interconnected mechanisms in the O\textsubscript{2} control of STB development and therefore bring new perspectives towards the pathogenesis of pregnancy complications such as PE and FGR.

1.11. Hypothesis

I hypothesize that hypoxia/HIF signaling impairs placental STB formation by regulating transcription of specific genes required for CTB differentiation and fusion.

1.12. Thesis Aims

\textit{Aim 1}: To determine the effects of different O\textsubscript{2} concentrations on STB formation

\textit{Aim 2}: To characterize the role of hypoxia/HIF signaling in regulating STB formation

\textit{Aim 3}: To identify downstream targets of HIF that may control STB formation
Chapter 2: Methods

2.1. Cell Culture and Treatments

Human choriocarcinoma-derived BeWo cells (frequently used to model the villous CTB lineage) were obtained from American Type Culture Collection (CCL-98, Manassas, VA, USA). Cells were tested for mycoplasma contamination to ensure reliability and consistency across passages, and were cultured in standard growth media composed of DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µM streptomycin. Cells were maintained in a humidified cell culture chamber set to 37 °C and 5% CO₂, with the remaining atmosphere consisting of room air (containing approximately 20% O₂). Henceforth, this culture condition will be referred to as the standard or 20% O₂ condition. Cells were passaged through trypsinization prior to reaching 80-90% confluency and were maintained for no more than 20 consecutive passages. To induce differentiation into STB-like cells in vitro, BeWo cells were cultured in standard growth media containing the membrane-permeable cAMP derivative, 8-Bromo-cAMP (250 µM, B7880, Sigma-Aldrich, Oakville, ON, Canada) for 24 or 48 h, as we have done previously.¹²³

Human TS cells derived from 7-week human placenta (CT29 line) were cultured and maintained as previously described.⁴¹ Cells were passaged using TrypLE (ThermoFisher Scientific, Whitby, ON, Canada) prior to reaching 80-90% confluency and maintained at standard cell culture conditions for no more than 20 consecutive passages. For experiments, human TS cells were induced to differentiate into STB-like cells by culturing for 48 h in STB differentiation medium under 20% O₂ conditions, followed by an additional 48 h in a basal differentiation medium under 20%, 8%, or 1% O₂ conditions. STB differentiation medium was composed of DMEM/F12 supplemented with 0.1 mM 2-mercaptoethanol (21985-023, ThermoFisher Scientific), 0.5% Penicillin-Streptomycin (15140-122, ThermoFisher Scientific), 0.3% bovine serum albumin (BSA, A1595, Sigma-Aldrich), 1% ITS-X supplement (51500-056, ThermoFisher Scientific), 2.5 mM Y27632 (04-0012,
Stemgent, Cambridge, MA, USA), 2 mM forskolin (F6886, Sigma-Aldrich), and 4% knockout serum replacement (10828-028, ThermoFisher Scientific). Basal differentiation medium included all components of the STB differentiation medium except forskolin. Undifferentiated human TS cells were maintained as CTB-like cells on cell culture dishes coated with 5 µg/mL collagen IV (3410-010-02, Bio-Techne, Toronto, ON, Canada), while cells induced to differentiate towards STB were maintained on culture dishes coated with 2.5 µg/mL collagen IV.

Human embryonic kidney (HEK)-293T cells were obtained from American Type Culture Collection (CRL-3216) and were maintained in DMEM medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 µM streptomycin. Cells were passaged by light trypsinization prior to reaching 80-90% confluency and were maintained in a humidified cell culture chamber at standard culture conditions.

To assess the capacity of BeWo and human TS cells to differentiate into STB under various O₂ environments, cells were cultured in standard culture conditions (37 °C, 5% CO₂, ~20% O₂) or in two separate humidified cell culture chambers placed at 37 °C and flushed with a mixture of gases containing 5% CO₂ and 95% nitrogen to establish low O₂ conditions. O₂ levels in each low O₂ chamber were monitored using separate ProOx 110 control devices (Biospherix, Parish, NY, USA) and were set to approximately 8% or 1% O₂. To evaluate the O₂ gradient between the cell culture chambers at different O₂ levels, BeWo cells were grown to confluency and treated with 5 µM of the Image-IT Green Hypoxia Reagent (I14834, ThermoFisher Scientific) in cell culture medium for 30 min as per manufacturer’s instructions. The medium was then replaced with fresh medium, and cells were placed in the culture chambers at 20%, 8%, or 1% O₂ conditions for about 4 h. Fluorescence and brightfield images of the cells were subsequently taken using a Zeiss Axio fluorescence microscope equipped with a 10 × Plan-Neofluar objective (0.3 NA).

2.2. Immunofluorescence

Cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton-X-100, blocked using 10% normal goat serum (ThermoFisher Scientific), and incubated with a primary
antibody solution containing antibodies targeting ZO-1 (33-9100, 1:150, Life Technologies, Carlsbad, CA, USA) and hCG (PA5-16265, 1:500, Life Technologies). The following day, cells were incubated in a secondary antibody solution containing species-specific Alexa 488 and Alexa 555-conjugated fluorescent antibodies (ThermoFisher Scientific). Nuclei were then counterstained using 4’,6-diamidino-2-phenylindole (DAPI, ThermoFisher Scientific) and fluorescence images were taken using a Zeiss Axio fluorescence microscope equipped with a 20 × Plan-Neofluar objective (0.4 NA). For quantification of STB formation, three images from random fields per well were captured at 20 × magnification. Loss of ZO-1 staining combined with presence of hCG staining was used to identify clusters of cells that formed STB. Percent STB per image was calculated by dividing the number of nuclei within STB (ZO-1 negative and hCG positive) by the total number of nuclei in the field of view and multiplying by 100%. Triplicate values from the three images were then averaged to calculate the percent STB formed per condition. Nuclei were counted using the ImageJ software (version 1.52) and an in-house automated nuclei counting program designed in MATLAB.

2.3. Quantitative Reverse Transcription PCR

RNA was extracted from cells using RiboZol (Amresco, Mississauga, ON, Canada) as per manufacturer’s instructions and converted into cDNA through reverse transcription (High Capacity cDNA kit, ThermoFisher Scientific). The cDNA was then diluted 1:10 and quantitative RT-PCR (qRT-PCR) was performed using a CFX96 Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada), SensiFAST SYBR Lo-ROX PCR Master Mix (BIO-98050, FroggaBio, Toronto, ON, Canada), and primer sets indicated in Table 2.1. Cycling conditions involved an initial holding step (95 °C for 10 min), followed by 40 cycles of 2-step PCR (95 °C for 15 s and 60 °C for 1 min) and ending with a dissociation phase. Relative mRNA expression was calculated through the ΔΔCt method, using the geometric means of Ct values from three constitutively expressed reference genes (RNA18SNI, EEF2, and YWHAZ).
Table 2.1. Forward and reverse primers for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primers (FWD &amp; REV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERVFRD-1</td>
<td>NM_207582.3</td>
<td>FWD CCAATTTCCCTCCTCCTCCTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>REV CGGTTGTTAGTTTGGCTTGTGGT</td>
</tr>
<tr>
<td>OVOL1</td>
<td>NM_004561.4</td>
<td>FWD CCGTGCGTCTCCACGTGCAAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>REV GCTGCTGCTGCGAGAGGCA</td>
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<td>CGB</td>
<td>NM_000737.3</td>
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<td></td>
<td></td>
<td>REV GCTTTATACCTCGGCTGGTTG</td>
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<tr>
<td>HSD11B2</td>
<td>NM_000196.4</td>
<td>FWD CAGATGGACCTGACCAAAACC</td>
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<td></td>
<td></td>
<td>REV AGCTCCGCATCAGCAACTAC</td>
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<tr>
<td>HSD3B1</td>
<td>NM_000862.3</td>
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<td></td>
<td></td>
<td>REV CAGGCAACATTGGCCAACATA</td>
</tr>
<tr>
<td>TEAD4</td>
<td>NM_003213.4</td>
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<td></td>
<td>REV TCTTGGACTGTTGGATGGA</td>
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<td>GJA1</td>
<td>NM_000165.5</td>
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<td>ASCL2</td>
<td>NM_005170.3</td>
<td>FWD AACCTGAGCTGCTGGAGGGACA</td>
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<td>REV TCTTGGACGAGCGGAAAAACTC</td>
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<td>RNA18SN1</td>
<td>NR_145820.1</td>
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</tr>
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<td>EEF2</td>
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<td></td>
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<tr>
<td>YWHAZ</td>
<td>NM_003406.3</td>
<td>FWD ATGCAACCAACACATCCTATC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>REV GCATTATTAGCGTGCTGTCTT</td>
</tr>
</tbody>
</table>
2.4. Western Blotting

Cell lysates were prepared using a 1 × Laemmli sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, 0.125 M Tris-HCl and 62.5 mM dithiothreitol) supplemented with phenylmethylsulfonyle fluoride, sodium fluoride, sodium orthovanadate, and protease inhibitor cocktail (Sigma-Aldrich). Samples were then boiled for 5 min, loaded onto SDS-containing polyacrylamide gels, and proteins were separated using SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride membranes, blocked using tris buffered saline containing 3% BSA and 0.1% Tween-20, and probed with antibodies targeting ARNT (5537, 1:1000, Cell Signaling Technology, Danvers, MA, USA), hCG (PA5-16265, 1:1000, Life Technologies), TEAD4 (HPA056896, 1:200, Sigma-Aldrich) and ACTB (sc47778, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The following day, membranes were incubated with species-appropriate secondary antibodies, and signals were detected using a LI-COR Odyssey imaging system (LI-COR, Lincoln, NE, USA).

2.5. Flow Cytometry

To assess viability of BeWo cells under different O₂ conditions, cells were cultured for 48 h in separate cell culture chambers at 20%, 8%, or 1% O₂. As a positive control, 1 µM camptothecin (Cell Signaling Technology) was also added to cells cultured under 20% O₂ for 24 h prior to detection. Cells were then trypsinized, centrifuged, and incubated with annexin V and propidium iodide (PI) as per the manufacturer’s instructions (Annexin V-FITC Apoptosis Detection Kit, 509297, ThermoFisher Scientific). Annexin V and PI positive cells were quantified via flow cytometry using a BD FACSCelesta Cell Analyzer equipped with a 488 nm blue laser and 561 nm yellow-green laser for detection of annexin V and PI, respectively (BD Biosciences, San Jose, CA, USA). Data were analyzed using the FlowJo software (version 10.8.1). The gating strategy commenced with a forward scatter area versus forward scatter height plot to eliminate doublets, followed by a second gate on forward scatter area versus side scatter area to remove cellular debris while retaining both live and dead cells (shown in Fig. 3.2).
2.6. Lentivirus Production and Transduction

To knockdown ARNT gene expression, two ARNT short hairpin RNA (shRNA) constructs encoded in PLKO.1 vectors (shRNA1 – TRCN0000003816 and shRNA2 – TRCN0000003819) were obtained from Sigma-Aldrich. A control PLKO.1 shRNA vector containing an shRNA that targets a non-mammalian transcript (green fluorescent protein; GFP) was obtained from Addgene (plasmid 12273, Addgene, Watertown, MA, USA). For the split GFP complementation assay, EF.GFP lentiviral vectors (17616, Addgene) expressing separate split GFP components were generated. One plasmid encoded the first to seventh β-sheets of GFP and the second plasmid encoded the eighth to eleventh β-sheets, as previously detailed. Lentivirus was produced using lentiviral packaging and envelope plasmids (MD2.G, MDLG/RRE, and RSV-Rev) as described previously. In brief, HEK-293T cells were transfected with the lentiviral plasmids along with the shRNA-containing or split-GFP containing vectors using Lipofectamine 2000 (ThermoFisher Scientific). Culture supernatants containing lentivirus were then collected every 24 h for a total of 48 h and were stored at -80 °C until further use. To transduce BeWo cells, cells were exposed to lentiviral particles in the presence of 8 μg/mL hexadimethrine bromide (Sigma-Aldrich) in cell culture media for 48 h. For transduction with shRNA-containing lentivirus, transduced cells were subsequently selected using puromycin (3.5 μg/mL, Sigma-Aldrich) for an additional 48 h.

2.7. Split GFP Complementation Assay

BeWo cells separately transduced with lentivirus carrying a complementary split GFP component were cocultured at a 1:1 ratio. The next day, the medium was replaced with cell culture medium containing 250 μM 8-Bromo-cAMP to induce fusion and differentiation into STB-like cells for 48 h. Fused cells were represented as GFP-positive areas where cytoplasmic mixing allowed the split GFP components to self-reassociate (see Fig. 2.1). To demonstrate the assay, the GFP fluorescence during fusion was monitored through live-cell imaging of the BeWo cells maintained at standard cell culture conditions (37 °C, 5% CO2, ~20% O2) in an enclosure underneath a Nikon fluorescence microscope equipped with a 10 × Plan Apo Lambda objective (0.45 NA). Time-lapse fluorescence and brightfield
images of cells in culture were recorded at $10 \times$ magnification every 5 min for up to 48 h, with image acquisition controlled using the NIS-Elements AR software (version 4.30.02). For experiments assessing BeWo cell fusion under different O$_2$ conditions, cocultured split GFP-expressing cells plated into at least four wells of a 96-well plate were treated with 8-Bromo-cAMP for 48 h in separate cell culture chambers at 20%, 8%, or 1% O$_2$. Following 48 h of induced differentiation, a Zeiss Axio fluorescence microscope equipped with a 20 × Plan-Neofluar objective (0.4 NA) was used to capture a single, random image from each of the 4 wells. Using ilastik machine learning software, GFP-positive regions in the images were classified, and image segmentation was performed to generate black and white images denoting GFP-positive and GFP-negative regions, respectively$^{128}$. ImageJ software was then used to calculate the percent GFP-positive area in the images to serve as the fusion index$^{124}$, and values from each of the 4 wells were subsequently averaged to calculate the percent fusion per condition.

Figure 2.1. Schematic of the split GFP complementation assay to quantify cell fusion.
2.8. HRE/HIF Reporter Assay

To assess HIF transcriptional activity, the pGL4.42[luc2P/HRE/Hygro] plasmid, which contains four copies of an HRE/HIF binding site that drives transcription of the firefly luciferase reporter gene luc2P (E4001, Promega, Madison, WI, USA) was obtained. The plasmid was transfected into BeWo cells using jetPRIME (Polyplus Transfection, Illkirch-Graffenstaden, France), as previously described. Briefly, 1.5 µg of the vector was added to 200 µL of jetPRIME buffer, mixed with 2.5 µL jetPRIME transfection reagent, and incubated for 10 min. The transfection mix was then added dropwise to wells with wildtype BeWo cells or BeWo cells stably expressing control/ARNT-specific shRNA. Cells were then incubated in separate cell culture chambers at 20%, 8%, or 1% O₂ conditions for 48 h, lysed using 1 × Glo Lysis Buffer (E2661, Promega), and subsequently treated with Bright-Glo™ Luciferase Assay Reagent (E2610, Promega). Firefly luciferase activity was measured using a Perkin Elmer VICTOR³V luminescence microplate reader.

2.9. Enzyme-Linked Immunosorbent Assay

Levels of progesterone in supernatants of wildtype BeWo cells cultured under 20%, 8%, or 1% O₂ were measured via an enzyme immunoassay (EIA-1561, VWR International, Mississauga, ON, Canada), using a protocol provided by the manufacturer. In brief, media was collected from BeWo cells, centrifuged to remove cellular debris, and supernatants were added to a microplate precoated with a polyclonal antibody targeting progesterone. A solution of enzyme conjugate (progesterone conjugated to horseradish peroxidase) was then added to compete with progesterone from the samples for binding to the coated antibody. After a washing step, a substrate solution containing tetramethylbenzidine was added to the microplate to initiate a colorimetric reaction, which was ultimately stopped following addition of a stop solution containing 0.5 M sulfuric acid. The absorbance of the resulting solution was measured, a standard curve was generated by plotting absorbance values against defined concentrations of standards, and concentrations of progesterone in BeWo supernatants were determined using this standard curve. BeWo media was diluted in the appropriate buffer prior to performing the assay to ensure that absorbance values fell within the linear range of the standard curve. The sensitivity of the ELISA is 0.045 ng/mL.
2.10. EdU Cell Proliferation Assay

A 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay was conducted to measure cell proliferation of BeWo cells stably expressing control or ARNT-specific shRNA and was performed according to the manufacturer’s instructions (Click-iT EdU Cell Proliferation Assay, ThermoFisher Scientific). In brief, 10 μM EdU was added to BeWo cell media and provided to cells for 48 h. Following incubation, cells were fixed in 4% paraformaldehyde, permeabilized using 0.3% Triton-X-100, and incubated with Click-iT EdU reaction cocktail for 30 min. Nuclei were then stained using Hoechst 33342, and cells were imaged using a Zeiss Axio fluorescence microscope equipped with a 20 × Plan-Neofluar objective (0.4 NA). Nuclei were counted using ImageJ and MIPAR Image Analysis Software (version 3.4.1.0)\textsuperscript{124,130}.

2.11. Chromatin Immunoprecipitation Sequencing

Chromatin Immunoprecipitation (ChIP) was performed as previously described\textsuperscript{123}. BeWo cells cultured under 1% O\textsubscript{2} for 24 h were fixed with 0.7% formaldehyde for 10 min, lysed, and sonicated using a Bioruptor (Diagenode, Denville, NJ, USA) to generate DNA fragments less than 400 bp. Approximately 1% of sonicated nuclear lysate was removed to serve as an input control. Half of the remaining lysate was incubated with ARNT antibody (2 μg, 5537, Cell Signaling Technology) and the other half was incubated with a negative control rabbit IgG (2 μg, 2729, Cell Signaling Technology) overnight at 4 ºC. The next day, immunoprecipitated chromatin fragments were captured using protein G-conjugated Sepharose beads (Sigma-Aldrich), and DNA fragments were eluted and purified. Aliquots of input and ChIP DNA were then assessed using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and quantified using Qubit. Libraries were generated using a NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, Whitby, ON, Canada) and sequenced using an Illumina NextSeq 500 System (Illumina, San Diego, CA, USA) at the London Regional Genomics Facility using 75-bp paired end reads, which generated about 30 million reads per sample.
The majority of ChIP sequencing (ChIP-seq) analysis was performed using the online Galaxy platform\textsuperscript{131}. Raw sequence reads that passed quality control filtering were mapped onto the hg38 human reference genome downloaded from the UCSC genome browser\textsuperscript{132} using BWA (version 0.7.17)\textsuperscript{133}. Blacklisted regions\textsuperscript{134}, PCR duplicates, and multimapping reads with a MAPQ quality score below 20 were then filtered out. Peaks were called for three independent biological replicates using MACS2 (version 2.1.1.2) in paired-end mode using default parameters\textsuperscript{135}, and a single set of reproducible peaks from all three replicates were then generated using ChIP-R (version 1.1.0)\textsuperscript{136}. For peak visualization, RPKM normalized coverage tracks were generated from aligned reads using the bamCoverage tool in deepTools (version 3.3.2)\textsuperscript{137}, and visualized using the Integrated Genome Viewer software (version 2.12.2). Motif analysis of sequences enriched under ChIP-seq peaks was done using the \textit{de novo} motif discovery platform MEME-ChIP\textsuperscript{138}. A 400 bp sequence surrounding each of the peak summits (extending 200 bp on either side) was entered into MEME-ChIP as input and analyzed using default settings for motif width and significance thresholds. Gene Ontology (GO) term enrichment for biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of genes associated with ChIP-seq peaks were generated using the clusterProfiler package in R\textsuperscript{139}. Raw and processed data are available on NCBI’s Gene Expression Omnibus website (accession no. GSE209936)\textsuperscript{140}.

\textbf{2.12. Statistical Analysis}

Statistical analysis for ChIP-seq was described previously in section 2.11. Statistical comparisons between three or more means were performed using a one or two-way ANOVA (for one or two independent variables, respectively), followed by Holm-Sidak’s multiple comparisons test. In two-way ANOVA analyses, $P_{\text{int}}$ represents the P-value for the interaction between $O_2$ levels and differentiation condition. All experiments were repeated at least three independent times. Means were considered statistically different if the P-value was less than 0.05. GraphPad Prism 8.0 was used for all graphing and statistical analyses.
Chapter 3: Results

3.1. STB Formation is Impaired in BeWo Cells Under Low O$_2$

To determine the effect of different O$_2$ tensions on STB formation, CTB-like cells of the BeWo choriocarcinoma cell-line were induced to differentiate into STB for 48 h using the cAMP analogue 8-Bromo-cAMP under 20%, 8%, or 1% O$_2$ conditions. The 1% O$_2$ condition was intended to reflect the low O$_2$ placental environment in early pregnancy (that is also thought to aberrantly persist in PE/FGR), while the 8% O$_2$ condition was intended to mirror the physiologically “normoxic” placental environment after the twelfth week of gestation. The 20% O$_2$ condition represented standard cell culture conditions. The ability of cells to form STB under each O$_2$ environment was compared using immunofluorescence to quantify the percentage of multinucleated, hormone-producing STB formed (detected by loss of ZO-1 staining and presence of hCG staining between nuclei), as well as qRT-PCR and western blotting to assess expression of CTB and STB markers. When compared to standard (20% O$_2$) conditions, there was a 72% and 82% decrease in STB formation under 8% and 1% O$_2$ conditions, respectively, as determined by immunofluorescence staining (Fig. 3.1 A and B, N=3, P<0.05). BeWo cells induced to differentiate for 48 h under 8% and 1% O$_2$ also showed decreased expression of STB marker genes, including ERVFRD-1, OVOL1, CGB, and HSD11B2 (Fig. 3.1C, N=4, all P<0.05). Furthermore, there were increased mRNA and protein levels of the CTB marker TEAD4 as well as reduced protein levels of the STB hormone hCG under 8% and 1% O$_2$ when compared to 20% O$_2$ conditions (Fig. 3.1 D and E, N$\geq$3, all P<0.05). Thus, when cultured under 8% and 1% O$_2$, BeWo cell differentiation and fusion is impaired.

To verify that BeWo cells cultured under 8% and 1% O$_2$ conditions were indeed exposed to lower O$_2$ availability, cells were treated with the Image-iT Green Hypoxia Reagent, a live-cell permeable compound that becomes fluorescent under decreased O$_2$. Cells were then incubated for 4 h under 20%, 8%, or 1% O$_2$, and imaged using a fluorescence microscope. As expected, there was an increase in green fluorescence as the O$_2$ levels
decreased, although there was also some signal apparent in the 20% O₂ condition (Fig. 3.2A). To determine whether BeWo cells cultured at 8% and 1% O₂ activate HIF signaling in response to low O₂, cells were transfected with a pGL4.42[Luc2P/HRE/Hygro] plasmid containing four copies of an HRE that drives transcription of a luciferase reporter gene, followed by subsequent 48 h culture under each O₂ condition. Compared to the 20% O₂ condition, luciferase activity was increased in transfected cells cultured under 8% and 1% O₂, suggesting greater activation of HREs by HIF during culture under lower O₂ levels (Fig. 3.2B, N=3, P<0.05). To ensure that culture under low O₂ did not alter BeWo cell viability, annexin V and PI staining was assessed via flow cytometry following 48 h culture under each O₂ condition. As a positive control, cells cultured under 20% O₂ were also treated with 1 µM camptothecin in cell culture media for 24 h prior to detection. While there was a much greater percentage of annexin V and PI-positive cells following exposure to camptothecin (approximately 25% of cells), untreated cells cultured under 20%, 8% or 1% O₂ showed comparable annexin V and PI positivity (approximately 3-4% in each group, Fig. 3.2 C-E, N=3, P<0.05). These results demonstrated that culture under 8% and 1% O₂ impairs STB formation in BeWo cells through a mechanism independent of altered cell viability.
BeWo cells were cultured for 48 h in the presence or absence of differentiation conditions under 20%, 8%, or 1% O₂. (A and B) Representative immunofluorescence images (A) and percent STB (B), calculated as the number of nuclei within syncytia divided by the total number of nuclei present (×100%; N=3). ZO-1 negative and hCG positive staining denotes cells that fused to form STB. DAPI was used to highlight nuclei. (C and D) qRT-PCR analysis of differentiation (C, N=4) and stem (D, N=5) markers. (E) Western blot analysis of CTB-associated TEAD4 and STB-associated hCG protein levels (N=3). ACTB was used as a loading control for the western blot. Hash marks (#) above bars represent groups in the differentiation condition significantly different (P<0.05) from 20% O₂ differentiated cells, using two-way ANOVA followed by Holm-Sidak’s multiple comparisons test. Graphs represent means (SEM). Scale bars = 50 μm.

Figure 3.1. STB formation is impaired in BeWo cells under low O₂.
**Figure 3.2.** Validation of low O$_2$ culture and assessment of cell viability in BeWo cells. 
(A) Brightfield and corresponding fluorescence images of BeWo cells treated with the Image-iT Green Hypoxia Reagent and incubated for 4 h under 20%, 8% or 1% O$_2$. (B) Relative luminescence of cells transfected with a pGL4.42[luc2P/HRE/Hygro] vector and subsequently cultured for 48 h under each O$_2$ condition. Asterisks (*) above bars represent groups significantly different (N=3, P<0.05) from 20% O$_2$ cultured cells, while hash marks (#) above bars represent groups significantly different from one another, using one-way ANOVA followed by Holm-Sidak’s multiple comparisons test. (C-E) Representative dot plots (C) and percentage of annexin V and PI-positive cells (D) from flow cytometry analysis of cells cultured under 20%, 8%, or 1% O$_2$ for 48 h, or treated with 1 µM camptothecin under 20% O$_2$ for 24 h. (E) Gating strategy used to exclude doublets and debris (graphs on the left and right, respectively). Asterisks (*) above bars represent groups significantly different (N=3, P<0.05) from untreated, 20% O$_2$ cultured cells, using one-way ANOVA followed by Holm-Sidak’s multiple comparisons test. Graphs represent means (SEM). Scale bars = 100 µm.
3.2. CTB Fusion into Functional STB is Impaired in BeWo Cells Under Low O₂

CTB fusion is an important step in the formation of functional STB. To quantify fusion, we developed a CTB fusion assay adapted from measuring viral-host cell fusion kinetics that employs pairs of complementary reporter proteins which can generate a quantifiable signal after cells fuse. This assay makes use of a split GFP that self-reassociates and fluoresces following fusion. For this technique, one population of BeWo cells were transduced with a plasmid expressing one split GFP component, and another identical population of cells were transduced with a plasmid expressing the complementary component. The two populations were then cocultured in a 1:1 ratio and fusion was quantified following 48 h of culture under 20%, 8%, or 1% O₂ by measuring the GFP fluorescence area, which occurred in regions where the split GFP components joined together following cytoplasmic mixing by cell fusion.

To demonstrate the split GFP fusion assay, time-lapse microscopy of cocultured BeWo cells expressing complementary split GFP components was performed over 48 h of 20% O₂ culture in the presence or absence of differentiation conditions. Only cells induced to differentiate using 8-Bromo-cAMP showed prominent GFP fluorescence, lending support to the use of this system in measuring CTB fusion (Fig. 3.3). Quantification of the percent GFP-positive area (serving as the fusion index) from fluorescence images taken after 48 h of induced differentiation under 20%, 8%, or 1% O₂ revealed a significant 48% and 75% reduction in cell fusion under 8% and 1% O₂, respectively, when compared to 20% O₂ (Fig. 3.4 A and B, N=3, P<0.05). To assess whether these findings paralleled a reduction in STB hormone secretory function, a progesterone ELISA was performed following induced differentiation of wildtype (non-transduced) BeWo cells for 48 h under 20%, 8%, or 1% O₂. Progesterone secretion into cell culture media was decreased by 74% and 96% for cells induced to differentiate under 8% and 1% O₂, respectively, when compared to cells cultured under 20% O₂ (Fig 3.4C, N=3, P<0.05). This corresponded with a 94% and 91% decreased mRNA expression of HSD3B1, which encodes a key steroidogenic enzyme involved in progesterone biosynthesis, for cells cultured under 8% and 1% O₂, respectively (Fig 3.4D, N=3, P<0.05).
Figure 3.3. Live-cell imaging of split GFP complementation assay to measure BeWo cell fusion.

Two populations of BeWo cells were separately transduced with a plasmid expressing a complementary split GFP component. Cells were then cocultured in a 1:1 ratio for 48 h in the presence or absence of differentiation conditions under 20% O₂. Brightfield and fluorescence images depict GFP-positive areas denoting cells that fused to form STB at several time points: 0 h, 16 h, 32 h, and 48 h. Scale bars = 100 μm.
**Figure 3.4.** CTB fusion into functional STB is impaired in BeWo cells under low O\textsubscript{2}.

(A and B) Representative fluorescence images (A) depicting GFP fluorescence of two populations of BeWo cells separately transduced with a plasmid expressing a complementary split GFP component and cocultured in a 1:1 ratio for 48 h in the presence or absence of differentiation conditions under 20\%, 8\%, or 1\% O\textsubscript{2}. Percent fusion (B) was calculated as percent GFP-positive area. (C and D) Wildtype BeWo cells were cultured for 48 h in the presence or absence of differentiation conditions under 20\%, 8\%, or 1\% O\textsubscript{2}. Progesterone was measured in cell culture media by ELISA (C) and the relative expression of *HSD3B1* was assessed through qRT-PCR analysis (D). Asterisks (*) above bars represent groups in the undifferentiated condition significantly different (N=3, P<0.05) from 20\% O\textsubscript{2} undifferentiated cells, while hash marks (#) above bars represent groups in the differentiation condition significantly different from 20\% O\textsubscript{2} differentiated cells, using two-way ANOVA followed by Holm-Sidak’s multiple comparisons test. Graphs represent means (SEM). Scale bars = 50 \(\mu\text{m}\).
3.3. STB Formation is Impaired in Human TS Cells Under Low O₂

Next, we sought to determine the effect of different O₂ tensions on STB formation using human TS cells derived from first-trimester placentas, which can be maintained as CTB-like cells or stimulated to form STB or EVT-like cells. Specifically, human TS cells were cultured in STB differentiation media for 48 h under 20% O₂ to initiate the differentiation process, followed by culture in basal differentiation media for 48 h under 20%, 8%, or 1% O₂. The capacity of human TS cells to differentiate when cultured under each O₂ condition was compared using immunofluorescence to assess STB formation as well as qRT-PCR and western blotting to assess expression of CTB and STB markers. Since low O₂ is also thought to stimulate CTB differentiation into EVTs, and human TS cells are capable of differentiating into EVT-like cells, expression of EVT markers were also evaluated.

Qualitatively, there was a noticeable reduction in the presence of ZO-1 negative, hCG positive STB when cells were cultured under 8% and 1% O₂, compared to cells cultured at 20% O₂ (Fig. 3.5A). There was also a decrease in the presence of microvilli characteristic of STB (indicated by red arrows) and an increase in the presence of intercellular bridges (indicated by red arrowheads) as the O₂ concentration decreased (Fig. 3.5B). Furthermore, human TS cells cultured under 8% and 1% O₂ conditions had decreased expression of STB marker genes, including ERVFRD-1, CGB, HSD11B2, and HSD3B1, when compared to cells cultured under 20% O₂ (Fig. 3.5C, N=3, all P<0.05). On the other hand, cells cultured under 8% and 1% O₂ conditions also showed increased expression of both CTB and EVT marker genes when compared to cells cultured under 20% O₂. This included a 3.15-fold and 2.88-fold increased expression of the CTB-associated genes TEAD4 and GJA1, respectively, as well as a 3.51-fold and 4.66-fold increased expression of the EVT-associated genes HLA-G and ASCL2, respectively, when cells were cultured under 1% O₂ conditions (Fig. 3.5 D and E, N=3, all P<0.05). Western blotting also revealed increased TEAD4 and decreased hCG protein levels for cells cultured under 1% O₂ relative to 20% O₂ (Fig. 3.5F, N=3, both P<0.05). Collectively, these results illustrated an impaired capacity for CTB-like cells, including BeWo and human TS cells, to form STB-like cells under low O₂ environments.
Figure 3.5. STB formation is impaired in human TS cells under low O₂.

(A and B) Immunofluorescence (A) and phase-contrast (B) images of human TS cells cultured for 48 h in STB differentiation media under 20% O₂ to initiate the differentiation process, followed by 48 h culture in basal differentiation media under 20%, 8%, or 1% O₂. ZO-1 negative and hCG positive immunostaining denotes cells that formed STB, with a DAPI counterstain highlighting nuclei. Red arrows indicate presence of microvilli, while arrowheads indicate presence of intercellular bridges. (C – E) qRT-PCR analysis of STB (C), CTB (D), and EVT (E) markers. (F) Western blot analysis of CTB-associated TEAD4 and STB-associated hCG protein levels, with ACTB used as a loading control. Asterisks (*) above bars represent groups significantly different (N=3, P<0.05) from 20% O₂ cultured cells using one-way ANOVA followed by Holm-Sidak’s multiple comparisons test. Graphs represent means (SEM). Scale bars = 50 μm.
3.4. Knockdown of ARNT Restores STB Formation Under Low O₂

To assess whether HIF signaling under low O₂ is responsible for the inhibition of STB formation, BeWo cells were transduced with lentivirus carrying shRNA targeting ARNT to generate ARNT knockdown cells (ARNT KD1 and KD2) as well as lentivirus carrying a control shRNA to generate control cells (CTRL). ARNT, or HIF1β, is the common and constitutively expressed subunit of the heterodimeric HIF transcription factor complex, so knockdown of ARNT results in deficiency of HIF signaling. Using this strategy, I successfully knocked down ARNT transcript expression by approximately 57% and 58% in ARNT KD1 and KD2, respectively, when compared to CTRL cells (Fig. 3.6A, N=3, P<0.05). ARNT protein expression was also reduced by approximately 63% and 77% in ARNT KD1 and KD2, respectively, when compared to CTRL cells (Fig. 3.6B, N=3, P<0.05). The capacity of CTRL and ARNT KD cells to form STB following 48 h of induced differentiation under 20%, 8%, and 1% O₂ was then assessed using immunofluorescence to quantify the percentage of ZO-1 negative, hCG positive STB formed, as well as qRT-PCR and western blotting for CTB and STB markers.

My results showed that the inhibitory effect of low O₂ on STB formation was prevented through knockdown of ARNT. Specifically, when cells were cultured under 1% O₂ in differentiation conditions, there was a 4.45-fold and 5.61-fold increase in STB formation for ARNT KD1 and KD2, respectively, compared to CTRL cells (Fig. 3.6 C and D, N=3, P<0.05). ARNT KD1 and KD2 also had increased expression of STB marker genes (ERVFRD-1, OVOL1, and CGB) when compared to CTRL cells cultured in differentiation conditions under 1% O₂ (Fig. 3.6E, N=4, P<0.05). In addition, western blotting showed decreased TEAD4 and increased hCG protein levels for ARNT KD1 and KD2 versus CTRL cells under 1% O₂ conditions (Fig. 3.6F, N=3, both P<0.05). A similar stimulatory effect on STB formation was observed when ARNT KD1 and KD2 cells were induced to differentiate for 48 h under 8% O₂ conditions (Fig. 3.7, N=3, P<0.05). Altogether, these results suggested that ARNT, likely as a key component of the HIF transcription factor complex, plays a role in the repression of STB formation under low O₂.
**Figure 3.6.** Knockdown of ARNT restores STB formation under 1% O\(_2\).

BeWo cells were transduced with lentivirus carrying a control shRNA (CTRL) or an shRNA targeting ARNT (ARNT KD1 and KD2) to knockdown ARNT expression. **(A and B)** Efficiency of ARNT knockdown was determined through qRT-PCR (A) to assess ARNT mRNA expression levels and western blotting (B) to assess ARNT protein levels. ACTB was used as a loading control for the western blot. Asterisks (*) above bars represent groups significantly different (N=3, P<0.05) from CTRL cells, while hash marks (#) above bars represent groups significantly different from one another, using one-way ANOVA followed by Holm-Sidak’s multiple comparisons test. **(C and D)** Representative immunofluorescence images (C) and percent STB (D) of CTRL, ARNT KD1, and ARNT KD2 cells cultured for 48 h in the presence or absence of differentiation conditions under 1% O\(_2\) (N=3). ZO-1 negative and hCG positive immunostaining denotes cells that formed STB, with a DAPI counterstain highlighting nuclei. **(E)** Analysis of STB differentiation markers in CTRL and ARNT KD cells using qRT-PCR (N=4). **(F)** Western blot analysis of CTB-associated TEAD4 and STB-associated hCG protein levels, with ACTB used as a loading control (N=3). Asterisks (*) above bars represent groups in the undifferentiated condition significantly different (P<0.05) from CTRL undifferentiated cells, while hash marks (#) above bars represent groups in the differentiation condition significantly different from CTRL differentiated cells, using two-way ANOVA followed by Holm-Sidak’s multiple comparisons test. Graphs represent means (SEM). Scale bars = 50 μm.
Figure 3.7. Knockdown of ARNT restores STB formation under 8% O₂.

(A and B) Representative immunofluorescence images (A) and percent STB (B) of CTRL, ARNT KD1, and ARNT KD2 cells cultured for 48 h in the presence or absence of differentiation conditions under 8% O₂. ZO-1 negative and hCG positive immunostaining denotes cells that formed STB, with a DAPI counterstain highlighting nuclei. (C) qRT-PCR analysis of differentiation markers in CTRL and ARNT KD cells. (D) Western blot analysis of CTB-associated TEAD4 and STB-associated hCG protein levels, with ACTB used as a loading control. Asterisks (*) above bars represent groups in the undifferentiated condition significantly different (N=3, P<0.05) from CTRL undifferentiated cells, while hash marks (#) above bars represent groups in the differentiation condition significantly different from CTRL differentiated cells, using two-way ANOVA followed by Holm-Sidak’s multiple comparisons test. Graphs represent means (SEM). Scale bars = 50 μm.
3.5. Knockdown of ARNT Promotes STB Formation Under Standard O$_2$

Interestingly, the experiments with CTRL and ARNT KD cells under 20% O$_2$ revealed that knockdown of ARNT in BeWo cells promotes STB formation even under standard cell culture O$_2$ conditions, suggesting that there may be a basal level of HIF signaling restricting differentiation in these environments. Using immunofluorescence staining to quantify the percentage of CTRL and ARNT KD cells that formed STB following 48 h of induced differentiation under 20% O$_2$, there was a significant 1.60-fold and 1.53-fold increase in STB formation for ARNT KD1 and KD2, respectively, compared to CTRL cells (Fig. 3.8 A and B, N=3, P<0.05). Similarly, increased expression of STB marker genes was evident in ARNT KD1 and KD2 when compared to CTRL cells induced to differentiate under 20% O$_2$. This included a 10.18-fold, 9.13-fold, and 4.35-fold increased *ERVFRD-1*, *OVOL1*, and *CGB* expression, respectively, in ARNT KD2 (Fig. 3.8C, N=3, all P<0.05). Furthermore, TEAD4 protein levels were decreased (in ARNT KD1 only), whereas levels of hCG were increased in both ARNT KD1 and KD2 when compared to CTRL cells induced to differentiate under 20% O$_2$ (Fig. 3.8D, N=3, P<0.05).

Consistent with the notion that ARNT inhibits BeWo cell differentiation and formation of STB-like cells, there was also increased STB formation and expression of STB marker genes in ARNT KD cells even under undifferentiated conditions within the various O$_2$ environments tested (Fig. 3.6, Fig. 3.7, and Fig. 3.8). Furthermore, during routine cell culture, ARNT KD cells consistently showed reduced proliferative capacity (reflecting decreased CTB self-renewal), which was corroborated by performing an EdU incorporation assay following addition of EdU to cell culture media for 48 h under 20% O$_2$. The percentage of ARNT KD2 that incorporated EdU was 36% less than CTRL cells, demonstrating that ARNT KD2 had a decreased proliferation rate, although there was no statistically significant difference in EdU incorporation between ARNT KD1 and CTRL cells (Fig. 3.9 A and B, N=3, P<0.05). These seemingly contradictory results between ARNT KD1 and KD2 may be due to the extent of ARNT knockdown (and thus how much HIF activity is repressed in these cells). To assess HIF activation of HREs in CTRL and ARNT KD cells, cells were transfected with the pGL4.42[luc2P/HRE/Hygro] vector and subsequently cultured for 48 h under 1% and 20% O$_2$ conditions. As expected, both ARNT
KD1 and KD2 exhibited decreased luciferase activity compared to CTRL cells when cultured under 1% O\textsubscript{2} (when HIF activity is maximal). However, ARNT KD2 showed a 57% reduction in luciferase activity and thus HRE activation compared to ARNT KD1. This suggested that ARNT KD2 had a stronger inhibitory effect on HIF transcriptional activity relative to ARNT KD1, which most likely explains the decreased proliferation rate and increased expression of STB marker genes observed in ARNT KD2. Notably, HRE activation was also reduced through ARNT knockdown under 20% O\textsubscript{2}, since there was an 80% and 95% decrease in luciferase activity for ARNT KD1 and KD2, respectively, compared to CTRL cells (Fig 3.9C, N=3, P<0.05). Overall, these results suggested that ARNT knockdown alone may promote STB formation in BeWo cells even under standard cell culture O\textsubscript{2} conditions, and that this is likely due to a basal level of HIF signaling.
Figure 3.8. Knockdown of ARNT promotes STB formation under 20% O₂.

(A and B) Representative immunofluorescence images (A) and percent STB (B) of CTRL, ARNT KD1, and ARNT KD2 cells cultured for 48 h in the presence or absence of differentiation conditions under 20% O₂. ZO-1 negative and hCG positive immunostaining denotes cells that formed STB, with a DAPI counterstain highlighting nuclei. (C) qRT-PCR analysis of differentiation markers in CTRL and ARNT KD cells. (D) Western blot analysis of CTB-associated TEAD4 and STB-associated hCG protein levels, with ACTB used as a loading control. Asterisks (*) above bars represent groups in the undifferentiated condition significantly different (N=3, P<0.05) from CTRL undifferentiated cells, while hash marks (#) above bars represent groups in the differentiation condition significantly different from CTRL differentiated cells, using two-way ANOVA followed by Holm-Sidak’s multiple comparisons test. Graphs represent means (SEM). Scale bars = 50 μm.
Figure 3.9. Knockdown of ARNT reduces cell proliferation and HIF activity.

(A and B) Representative fluorescence images (A) of incorporated EdU used to assess proliferation of CTRL, ARNT KD1, and ARNT KD2 cells, with Hoechst used to detect nuclei. (B) Percentage of EdU-positive nuclei. (C) Relative luminescence of CTRL and ARNT KD cells transfected with a pGL4.42[luc2P/HRE/Hygro] vector and subsequently cultured for 48 h under 1% and 20% O₂ conditions. A schematic of the plasmid is shown to the left of the graphs. Asterisks (*) above bars represent groups significantly different (N=3, P<0.05) from CTRL cells, while hash marks (#) above bars represent groups significantly different from one another, using one-way ANOVA followed by Holm-Sidak’s multiple comparisons test. Graphs represent means (SEM). Scale bars = 50 μm.
3.6. ChIP-seq Identifies Putative STB-Regulatory Targets of ARNT/HIF

To uncover the downstream targets of HIF involved in the repression of STB formation under low O\textsubscript{2}, a genome-wide analysis of ARNT binding sites in the DNA was performed using ChIP-seq on chromatin derived from BeWo cells cultured under 1% O\textsubscript{2} for 24 h. Peaks were called from ChIP-seq data for three independent biological replicates using the MACS2 algorithm\textsuperscript{135}, and only reproducible peaks from all three replicates (generated using ChIP-R\textsuperscript{136}) were used for downstream analyses. This led to the identification of 1,812 peaks genome-wide that were common across all three biological replicates, of which 562 were statistically significant with a P-value cutoff of P<0.01. Of these 562 peaks, about 38% appeared in either introns or distal intergenic regions, but the majority (58%) of the peaks were localized in promoters, consistent with the role of ARNT as a binding partner in the HIF transcription factor complex (Fig. 3.10A). To assess the validity of the ARNT ChIP-seq peaks, \textit{de novo} motif analysis was conducted using MEME-ChIP\textsuperscript{138}. The top motif identified shared similarity with known ARNT, HIF1\(\alpha\), and HIF2\(\alpha\) motifs (motif enrichment E-value = 2.7e-281), suggesting that the peaks well represented HIF binding sites across the genome (Fig. 3.10B). As an additional validation, central motif enrichment analysis was performed using the Centrimo algorithm, which analyzes the distribution of enriched motifs relative to ChIP-seq peak summits\textsuperscript{144}. Centrimo results showed that known ARNT, HIF1\(\alpha\), and HIF2\(\alpha\) motifs were centrally enriched near the summits of ARNT ChIP-seq peaks, with motif enrichment E-values of 5.9e-177, 3.2e-160, and 1.5e-182, respectively (Fig. 3.10C).

To elucidate the biological functions of the top 562 genes enriched with ARNT peaks, functional enrichment analyses were performed using the clusterProfiler package in R\textsuperscript{139}. GO term analysis revealed enrichment for genes that were mainly involved in responses to decreased O\textsubscript{2} levels or hypoxia and various metabolic processes (Fig. 3.10D). KEGG pathway analysis of the same gene set revealed enrichment for pathways involved in bacterial infection, cancers, as well as the Rap1, Ras, HIF-1, and FoxO signaling pathways (Fig. 3.10E). These findings supported the primary role of HIF in regulating genes that help cells to adapt and cope under low O\textsubscript{2} environments. However, among the 1,812 peaks generated in the ARNT ChIP-seq dataset, several peaks were also located within the
promoter regions (≤1 kb from the transcription start site) of genes with well-established roles in regulating placental STB formation. This included ERVH48-1, which encodes a known inhibitor of CTB fusion into STB\textsuperscript{145}, as well as OVOL1, which encodes a well-characterized transcription factor that facilitates CTB differentiation\textsuperscript{32}. Although the peak in the OVOL1 promoter did not reach statistical significance (P=0.1) in the dataset when all replicates were combined (likely due to variability in peak signal), it was significant in each individual biological replicate (P<0.05). Other genes, such as ZNF292 and BHLHE40 that have been shown to be upregulated during BeWo syncytialization\textsuperscript{32}, but whose specific functional roles in this process have yet to be elucidated, were among the top 562 genes in the ARNT ChIP-seq dataset and also had peaks within promoter regions (Fig. 3.10F). To investigate changes in expression of these putative HIF target genes in response to differentiation and/or exposure to low O\textsubscript{2}, qRT-PCR was performed using BeWo cells induced to differentiate for 24 h under 20% or 1% O\textsubscript{2} conditions. While there was a significant 1.48 and 19.47-fold increased expression of ERVH48-1 and OVOL1, respectively, following induced differentiation under 20% O\textsubscript{2}, ERVH48-1 transcript levels further increased up to 3.2-fold and OVOL1 transcript levels did not change in response to induced differentiation under 1% O\textsubscript{2}, suggesting impairment of fusion and differentiation into STB. Furthermore, mRNA expression of ZNF292 and BHLHE40 increased 2.62 and 7.57-fold, respectively, following induced differentiation under 20% O\textsubscript{2}, but there were no significant differences between the undifferentiated and differentiation conditions under 1% O\textsubscript{2} (Fig. 3.10G, N=4, all P<0.05). While the consequences of these altered transcriptional patterns are not yet known, they may contribute to poor syncytialization. Altogether, these results suggested that transcription of genes important for CTB fusion and differentiation may be regulated by HIF, which could explain the deficient STB formation observed under low O\textsubscript{2}. 
Figure 3.10. ChIP-seq analysis of ARNT binding sites identifies putative STB-regulatory HIF targets.

(A) Pie chart showing the proportion of the top 562 ARNT ChIP-seq peaks overlapping genomic features in BeWo cells. (B) Letter logo representation of the top motif identified using MEME-ChIP, which shared significant similarity with the ARNT, HIF1α, and HIF2α motifs. De novo motif analysis was performed using sequences within a [-200 bp, +200 bp] window around ARNT ChIP-seq summits. Letter logo size indicates nucleotide frequency and E-value represents the significance of motif enrichment compared to genomic background. (C) Graph generated using Centrimo, depicting the distribution of occurrence probabilities of motifs enriched under ARNT ChIP-seq peaks relative to ARNT-binding summits (i.e., position of best site in sequence). Central motif enrichment E-values are: 5.9e-177, 3.2e-160, and 1.5e-182, for ARNT, HIF1α, and HIF2α motifs, respectively. (D and E) Functional GO term (D) and KEGG pathway (E) enrichment analyses of genes associated with ARNT ChIP-seq peaks. ‘Count’ is the number of genes enriched in a given term and ‘GeneRatio’ is the percentage of total genes in the given term. (F) Representative genome browser views of RPKM-normalized ARNT binding profiles proximate to ERVH48-1, OVOL1, ZNF292, and BHLHE40, genes with well-established or potential roles in STB formation that also showed ARNT/HIF binding in the promoter regions. (G) qRT-PCR analysis of the targets shown in (F) using BeWo cells cultured for 24 h in the presence or absence of differentiation conditions under 20% or 1% O2. Asterisks (*) above bars represent groups significantly different (N=4, P<0.05) from 20% O2 undifferentiated cells, while hash marks (#) above bars represent groups significantly different from one another, using two-way ANOVA followed by Holm-Sidak’s multiple comparisons test. Graphs represent means (SEM).
Chapter 4: Discussion

STB forms the primary interface between the mother and fetus during pregnancy and performs several key placental functions. The surrounding O₂ environment is a critical determinant of STB formation, which may reflect physiological or pathological situations. In this thesis, I showed that culture of BeWo and human TS cells under 8% or 1% O₂ inhibits their differentiation and fusion into STB-like cells compared to cells cultured under standard (~20%) O₂ conditions. This was assessed via traditional molecular approaches as well as a fusion assay employing a split GFP complementation strategy with BeWo cells. I also demonstrated in BeWo cells that knockdown of ARNT, a key component of the HIF transcriptional complex, could restore STB formation under low O₂, suggesting that HIF signaling is likely responsible for the repressive effect of low O₂ on syncytialization. Furthermore, through ChIP-seq analysis, I identified putative downstream targets of HIF that may regulate STB formation under low O₂ and can be investigated in the future as possible avenues to treat STB dysfunction in pregnancy-related diseases associated with poor O₂ supply to the placenta.

Placental development during the first trimester of pregnancy normally occurs in a low O₂ environment. Measurements of the O₂ concentration within the intervillous space prior to termination of pregnancy indicated that the O₂ partial pressure is less than 20 mmHg (equivalent to 2-3% O₂) between 8-10 weeks of gestation. This low O₂ environment during early pregnancy is likely crucial for proper fetal and placental development, and it is thought to guide trophoblast lineage decisions, particularly towards the EVT lineage. For instance, when compared to standard cell culture O₂ conditions, culture under low O₂ inhibits first-trimester CTB syncytialization while promoting EVT development and expression of EVT markers (e.g., ASCL2), an effect mediated by HIF signaling and HIF-dependent downregulation of E-cadherin. Low O₂ culture also promotes first-trimester villous explant EVT outgrowth, expansion, and invasion, which supports the consensus that a low O₂ environment in early gestation potentiates the differentiation of CTBs along the EVT pathway. Studies using TS cells derived from mouse and rat
blastocysts reflect similar O₂ and HIF-dependent control of trophoblast cell lineage determination. Normally, rodent TS cells differentiate mostly into trophoblast cell lineages of the rodent placenta junctional zone, functionally analogous to the EVT cell columns arising from anchoring villi in the human placenta. However, cells deficient in ARNT or doubly deficient in HIF1α/HIF2α preferentially form trophoblast cell lineages of the labyrinth zone (including syncytialized trophoblasts), functionally equivalent to the STB in human. Culture of rodent TS cells under low O₂ also shifts development towards invasive trophoblasts, which is dependent on HIF signaling. In particular, HIF transcriptional activity downregulates E-cadherin as well as upregulates KDM3A (an epigenetic regulator that promotes a pro-invasive phenotype) and ASCL2 (a transcription factor essential for junctional zone development). My own experiments using human TS cells derived from first-trimester placentas support the role of O₂ in directing the trophoblast differentiation trajectory, as cells cultured under lower O₂ showed reduced syncytialization and expression of STB-specific markers (e.g., HSD11B2), as well as increased expression of CTB (e.g., TEAD4) and EVT (e.g., ASCL2) associated genes. A recent study by Hong et al. is consistent with these findings, as they similarly showed reduced CTB fusion and expression of STB markers for human TS cells cultured under 2% O₂ versus 20% O₂. Thus, it is likely that the low O₂ environment in the placenta during the first trimester normally restricts the extent of STB formation while favouring CTB proliferation and differentiation into EVTs. This would allow for an adequate maternal blood flow to the placenta as well as the maintenance of a CTB progenitor pool (capable of differentiating into STB) to support robust fetal growth in the second half of pregnancy.

As a consequence of the progressive dissolution of trophoblast plugs and remodeling of uterine spiral arteries by EVTs, maternal blood flow into the placental intervillous space is increased, resulting in a rise in O₂ tension to about 60 mmHg (~8% O₂) after approximately 12 weeks of gestation. However, failure of this transition to occur properly, as in poor vascular remodeling by EVTs, dysregulates blood flow and O₂ delivery to the intervillous space and may promote prolonged placental hypoxia that likely contributes to the poor STB formation observed in pregnancy diseases including PE or FGR. In this thesis, I showed that culture of BeWo cells under 1% O₂ inhibits syncytialization when compared
to standard (~20% O₂) conditions. This included reduced expression of genes crucial for CTB fusion and differentiation (ERVFRD-1, OVOL1), decreased hormone production and secretion (hCG, progesterone), and increased expression of stem markers (TEAD4). Impaired fusion of cells was also measured using a novel technique employing complementary parts of a split GFP that can self-reassociate and generate a quantifiable fluorescent signal following cell fusion. Notably, we observe similar outcomes for cells cultured under 8% O₂, which was originally intended to reflect the physiological placental environment in the second and third trimesters. Yet, this finding is in line with results of previous studies using primary term CTBs, wherein even a relatively modest reduction of O₂ levels to approximately 10% O₂ significantly impaired fusion into multinucleated STB and secretion of hormones including hCG, when compared to culture under ~20% O₂\(^2\). There are several potential reasons why in vitro syncytialization appears repressed under seemingly physiological O₂ tensions. To start, responses of cells to physiological O₂ exposures in vivo are accompanied and likely influenced by the dynamic intrauterine environment and complex maternal adaptations that are not present in a static in vitro culture system. It is also very difficult to accurately define the “normoxic” O₂ condition for trophoblasts in vivo. Past studies that measured blood O₂ levels in the placenta at various points in pregnancy are few in number with small samples sizes and high variability, and these measurements did not account for the majority of O₂ molecules in the blood transported by red blood cell hemoglobin. Thus, measurement of O₂ levels dissolved in the blood represents O₂ molecules that dissociated from hemoglobin and could simply represent a high consumption rate of O₂ by the placenta\(^85,152\). Furthermore, cell culture medium is devoid of O₂ carriers and is exposed to atmospheric gases, and the amount available to cells in culture will depend on the passive O₂ solubility\(^91\). Nevertheless, while in vitro models cannot completely mimic the in vivo conditions, they offer a means of studying the role of O₂ availability in trophoblast differentiation within a controlled environment.

Hypoxia-induced repression of STB formation likely contributes to the pathogenesis of PE and FGR, and accumulating evidence implicates HIF as having a key role in mediating this inhibitory effect. Colson et al. recently suggested that HIF2α contributes to the inhibition
of CTB fusion and differentiation in response to low O₂. Specifically, they demonstrated that siRNA silencing of HIF2α in primary term CTBs partially restores syncytialization under 2.5% O₂, and overexpression of a constitutively-active mutant version of HIF2α in BeWo cells represses biochemical differentiation even under 21% O₂ conditions. The authors also showed that HIF2α staining is increased in nuclei of STB from PE and FGR-affected placentas when compared to healthy control placentas. In this thesis, I elucidated the role of HIF in regulating STB formation by knocking-down HIF1β/ARNT, an approach that circumvented any possible compensation due to functional loss of particular HIF-α subunits. As predicted, I found that deficiency of ARNT (and therefore HIF signaling) could robustly restore STB formation and expression of key STB markers under both 1% and 8% O₂, suggesting that HIF indeed plays a role in the repression of STB development. Unexpectedly, I also discovered that knockdown of ARNT reduces HIF transcriptional activity (as measured using an HRE luciferase reporter plasmid) as well as promotes BeWo syncytialization even under 20% O₂ conditions. These findings suggested that a small degree of HIF signaling may be active in this environment and somewhat represses differentiation at a basal level. Indeed, previous studies have shown faint bands of HIF1α and HIF2α expression on western blots in control BeWo cells cultured under ~20% O₂. One explanation for this is that cells incubated in standard culture conditions (typically 5% CO₂ balanced with room air) may be exposed to a lower O₂ level than that in the incubation gas due to the diffusion limitations of O₂ through the culture media, especially for densely plated cultures with high metabolic activity. It is also important to acknowledge that HIF signaling can be induced by factors other than O₂ concentration, which may contribute to the activation of HIF in BeWo cells cultured at 20% O₂. Furthermore, ARNT interacts with other transcriptional regulators; for instance, ARNT dimerizes with the ligand-bound aryl hydrocarbon receptor to modulate xenobiotic metabolism. Further research investigating whether aryl hydrocarbon receptor signaling also regulates trophoblast differentiation and function is therefore warranted.

Using ChIP-seq, I identified sites in the genome where ARNT was bound in BeWo cells following 24 h of 1% O₂ culture. These sites were subsequently validated to represent HREs or HIF binding sites through de novo motif analysis and central motif enrichment.
analysis. While many of the genes enriched with the top ARNT ChIP-seq peaks were linked to terms and pathways reflecting adaptive responses to low O$_2$ as per functional enrichment analysis, I also found peaks within the promoter regions ($\leq$1 kb from the transcription start site) of genes with known roles in syncytialization ($ERVH48-1$, $OVOL1$). Sugimoto et al. previously identified $ERVH48-1$ as encoding a human ERV-derived, placenta-specific protein called suppressyn (SUPYN) that inhibits fusion of CTBs into STB, with anti-fusogenic effects specific to syncytin-1 but not syncytin-2 mediated syncytialization. In vitro experiments with human trophoblast cell-lines showed that both cell-associated and secreted SUPYN bound directly to the syncytin-1 receptor ASCT2 on fusing CTBs and thus blocked the binding of syncytin-1 expressed by STB to its receptor$^{156}$. The authors also showed that in primary CTBs from term placentas, SUPYN transcription, translation, and secretion were increased, while cell fusion was decreased when cells were cultured under lower O$_2$ conditions$^{145}$. This suggested that SUPYN is hypoxia-inducible and may contribute to impaired syncytialization under low O$_2$. Indeed, we confirmed in BeWo cells that the $ERVH48-1$ promoter is bound by ARNT/HIF, and its transcript levels are increased in response to low O$_2$. Furthermore, our group previously characterized OVOL1 as a key transcription factor that facilitates STB development by repressing genes encoding factors that maintain CTBs in a progenitor state, such as $MYC$, $ID1$, and $TP63^{32}$. In the current study, I showed using BeWo cells that ARNT/HIF binds the $OVOL1$ promoter and that induction of $OVOL1$ following induced differentiation is repressed under low O$_2$, likely a consequence of HIF transcriptional regulation. $ZNF292$ and $BHLHE40$ are two other putative HIF targets identified in the ChIP-seq dataset that were upregulated in response to induced differentiation under 20% O$_2$ but exhibited altered expression patterns under 1% O$_2$. Future work investigating expression of these factors under healthy and diseased states could provide new insight into the mechanisms of STB development and how it may be impaired in pregnancy complications linked to abnormal placental O$_2$ environments.

The present study has several strengths but is not without limitations. The use of the newly developed split GFP fusion assay in combination with a traditional immunofluorescence-based approach allowed for a comprehensive and robust analysis of the fusion index when BeWo cells were cultured under different O$_2$ conditions. In particular, the split GFP
technique could circumvent some limitations of counting fused cells manually, such as unintended observer bias, and it holds promise for increasing throughput of investigations for factors that may regulate STB formation. Yet, it is important to note that the fusion index represented by this assay is lower than the true value, since there is likely fusion between cells expressing the same split GFP component, which will not result in a quantifiable signal. Furthermore, this study made use of BeWo cells as the primary model. These cells can be induced to differentiate and fuse specifically into STB-like cells in a controlled manner upon treatment with 8-Bromo-cAMP, although they do possess genetic signatures quite distinct from that of normal trophoblast. Hence, human TS cells derived from first trimester placenta, which are genetically stable and show similarities in the transcriptome with villous CTBs, were used to corroborate select findings obtained using BeWo cells. Human TS cells also reliably differentiate into STB-like cells in a controlled manner following withdrawal of factors promoting the stem state and treatment with the adenylyl cyclase activator forskolin. Additional validation using other trophoblast cell models, such as primary CTBs isolated from placentas, would further strengthen this study. Moreover, it is important to recognize that the experimental systems used in this study do not completely reflect the physiological or pathological placental milieu. For instance, human TS cells are derived under room air, and BeWo cells have fully adapted to ambient O2 levels over many years; therefore, acclimatization of cells to lower O2 tensions (e.g., 8% O2) for multiple passages may improve representation of the models. Also, while I exposed cells to low O2 for 24 or 48 h to assess the role of HIF signaling in STB formation, evaluating trophoblast responses to intermittent hypoxia-reoxygenation is also valuable for future research, as this may better reflect the injuries to the placenta as a result of deficient spiral artery remodeling during pregnancy complications such as PE.

In conclusion, the current study shows that culture of CTB-like cells under low O2 impairs STB formation, which is likely mediated through HIF signaling. This inhibition may be relevant during normal placental development in the first trimester, when O2 levels are low (1-3% O2), to direct CTB cells towards the EVT lineage. However, repression of syncytialization after the twelfth week of gestation due to a failure to adequately increase O2 levels (up to ~8% O2) may have important contributions in the pathogenesis of
pregnancy diseases such as PE and FGR. Furthermore, several genes implicated in STB development (e.g., *ERVH48-1, OVOL1*) were found to have HIF binding in the promoters, suggesting that these genes may be HIF-regulated and that their altered expression under low O₂ are possible mechanisms underlying the deficient STB formation. Future studies should assess expression and localization of the STB-regulatory HIF targets in placentas from healthy versus pathological pregnancies, and evaluate whether modulating expression of these targets via selective inhibitors/activators could alleviate impaired STB formation in preclinical models of placental dysfunction. Therefore, this study opens doors to develop and test novel therapeutics that could improve prevention and/or management of PE and FGR, as well as increases our overall understanding of HIF-mediated gene regulation, which could be applicable to other cellular mechanisms beyond STB formation.
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