The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

Examining the Effects of Oxygen Tension on Extravillous Trophoblast Column Outgrowth During the First Trimester of Pregnancy

submitted by Jenna Elizabeth Treissman in partial fulfillment of the requirements for the degree of Master of Science in Reproductive and Developmental Sciences

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Abstract

Oxygen tension is thought to regulate many of the cellular and molecular processes that contribute to placenta development. An area of recent focus has been on the importance of a low-oxygen environment in controlling progenitor trophoblast differentiation along the invasive extravillous pathway. However, previously published research on the role of oxygen in trophoblast differentiation is conflicting. In this thesis, the effects of low (1%), physiologically normal (5%) and high (20%) oxygen conditions on extravillous trophoblast differentiation and column formation are examined using a human placental explant model. I show that culture in low oxygen conditions enhances column outgrowth and promotes the expression of pro-extravillous genes and gene pathways. By contrast, culture in high oxygen conditions promotes trophoblast proliferation, reduces column outgrowth and stalls differentiation along the extravillous pathway. I show that both low and physiologically normal oxygen conditions increase expression of the lysyl oxidase gene, and that this gene plays an important role in promoting extravillous column outgrowth. Together, these findings support hypoxia as an important factor driving trophoblast differentiation along the extravillous pathway. Additionally, this work provides new insight into specific molecular processes, regulated by oxygen tension, that may play an important role in early placentation.
Lay Summary

Healthy pregnancy depends on proper formation and function of the placenta, a temporary organ of pregnancy that facilitates transfer of oxygen, nutrients and waste between mother and baby. In early pregnancy, specialized cells of the placenta, called trophoblasts, acquire invasive characteristics and infiltrate the mother’s uterus. Trophoblast invasion is essential for establishing fetal access to maternal nutrients and maintaining pregnancy. Little is known about the molecular mechanisms that control this process, but oxygen is thought to play a role. The goal of my research is to study how low, moderate and high levels of oxygen control the development of invasive trophoblasts. Using a three-dimensional tissue culture system combined with imaging and non-biased gene measurement techniques, I show that exposure to low oxygen promotes the development of invasive trophoblasts. In contrast, exposure to high oxygen restrains this process. My research provides new insights into how oxygen conditions regulated placenta development.
Preface

This thesis is an original intellectual product of the author, Jenna Treissman, under the supervision of Dr. Alexander G. Beristain.

Dr. Alexander G. Beristain and I generated the hypothesis and experimental design for this work. Dr. Alexander G. Beristain performed the experiments described in chapters 2.4. Dr. Alexander G. Beristain and Jennet Baltayeva performed the experiments described in chapter 2.41. Dr. Alexander G. Beristain and I conducted the experiments described in chapter 2.6. I conducted all other experiments and analyses. Victor Yuan generated Figure 21 and Dr. Alexander G. Beristain generated Figures 22C and 24. I created all other figures. Jennet Baltayeva and Victor Yuan assisted in developing a workflow for gene microarray cleaning and analysis. I analyzed and interpreted the data with guidance from Dr. Alexander G. Beristain.

Tissue collection and experimentation were carried out in accordance with the guidelines of the Ethics Board on the Use of Human Subjects at the University of British Columbia [project number H13-00640].

A version of Chapters 3-5 of this thesis has been submitted for publication (Low oxygen enhances trophoblast column outgrowth by potentiating the extravillous lineage and promoting LOX activity. Jenna Treissman, Victor Yuan, Jennet Baltayeva, Hoa T. Le, Barbara Castellana, Wendy P. Robinson, Alexander G. Beristain). The co-authors contributed to the interpretation of the findings and critically revised the manuscript.

Raw and pre-processed gene expression data, along with phenotypic data for all samples used in the experiments described in this thesis have been submitted to the Gene Expression Omnibus (GEO), accession number GSE132421.
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<td>A disintegrin and metalloproteinase domain 12</td>
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<td>ADAM19</td>
<td>A disintegrin and metalloproteinase domain 19</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>BAPN</td>
<td>β-aminopropionitrile</td>
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<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<tr>
<td>CARE</td>
<td>Comprehensive Abortion and Reproductive Education</td>
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<td>CCNA2</td>
<td>cyclin A2</td>
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<td>CCTB</td>
<td>column cytotrophoblast</td>
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<td>caudal type homeobox 2</td>
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<td>conditioned media</td>
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<td>DAP</td>
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<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
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<td>dist CCTB</td>
<td>distal column cytotrophoblast</td>
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<tr>
<td>DMEM</td>
<td>dulbecco’s modified eagle media</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>endovascular extravillous trophoblast</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
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<td>egl-9 family hypoxia inducible factor 3</td>
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<td>endogenous retrovirus group W member 1, envelope</td>
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<td>FBS</td>
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<td>false discovery rate</td>
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<td>fms related tyrosine kinase 1</td>
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<td>GO</td>
<td>gene ontology</td>
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<td>HCG</td>
<td>human chorionic gonadotropin</td>
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<td>HELLP</td>
<td>haemolysis, elevated liver enzymes and low platelets</td>
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<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<td>HIF</td>
<td>hypoxia inducible factor</td>
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<td>HLA-G</td>
<td>human leukocyte antigen-G</td>
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<td>HPGH</td>
<td>human placental growth hormone</td>
</tr>
<tr>
<td>HPL</td>
<td>human placental lactogen</td>
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<tr>
<td>ID</td>
<td>patient identification</td>
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<tr>
<td>iEVT</td>
<td>interstitial EVT</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>ITGA5</td>
<td>integrin subunit alpha 5</td>
</tr>
<tr>
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<td>integrin subunit alpha 6</td>
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<td>IUGR</td>
<td>intrauterine growth restriction</td>
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<td>matrix metalloproteinase</td>
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<td>marker of proliferation Ki-67</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MYC</td>
<td>MYC proto-oncogene, bHLH transcription factor</td>
</tr>
<tr>
<td>NCAPH</td>
<td>non-SMC condensin I complex subunit H</td>
</tr>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PC</td>
<td>principal component</td>
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<tr>
<td>PCA</td>
<td>principal component analysis</td>
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<td>pre-SCT</td>
<td>pre-syncytiotrophoblast</td>
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<td>prox CCTB</td>
<td>proximal column cytotrophoblast</td>
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<td>PEG10</td>
<td>paternally expressed gene 10</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<td>PTB</td>
<td>preterm birth</td>
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<tr>
<td>RIN</td>
<td>RNA integrity number</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RORA</td>
<td>RAR related orphan receptor A</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<td>SGA</td>
<td>small for gestational age</td>
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<td>SPINT1</td>
<td>serine peptidase inhibitor, kunitz type 1</td>
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<td>syncytiotrophoblast</td>
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<td>TEAD4</td>
<td>TEA domain transcription factor 4</td>
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<tr>
<td>TFAP2A</td>
<td>transcription factor AP-2 alpha</td>
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<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>TIMP1</td>
<td>TIMP metallopeptidase inhibitor 1</td>
</tr>
<tr>
<td>TOP2A</td>
<td>DNA topoisomerase II alpha</td>
</tr>
<tr>
<td>UMAP</td>
<td>Uniform Manifold Approximation and Projection</td>
</tr>
</tbody>
</table>
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Firstly, I would like to express my sincere gratitude to my graduate supervisor, Dr. Alexander G. Beristain. His constant support, guidance and inspiration were instrumental to my learning and success. Thank you to my supervisory committee members Drs. Wendy Robinson and Julian Christians for their unwavering support, insightful suggestions and encouragement.

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I would like to extend my utmost gratitude to my family for their generosity and unconditional love. To my parents, thank you for supporting me in every way possible. Everything that I have accomplished is a direct result of your own hard work and loving care. You inspire and motivate me to achieve my goals.

Lastly, I would like to express my gratitude to the women who donated their placenta samples to my project. Thank you for using your abortion experience to make a meaningful contribution to the field of reproductive science. This research would not have been possible without your bravery and trust.
Dedication

I dedicate this research to my loving parents, Sharon Stevens and Dr. Simon Treissman.
CHAPTER 1. INTRODUCTION

1.1 Overview of the placenta

1.1.1 Functions

The placenta is a temporary and highly specialized organ of pregnancy that sustains fetal growth and development [1–4]. It achieves this through several critical functions including protection of the fetus from injury and infection, the facilitation of nutrient and gas exchange between maternal and fetal circulations, instruction of maternal immune tolerance toward fetal antigens and endocrine secretions required for the maintenance of pregnancy [1,5]. The placenta physically anchors the fetus to the mother’s uterus and establishes the physiological interface between maternal and fetal systems [6]. In the human placenta, chorionic villi are directly bathed in maternal blood. This close contact system, along with high oxygen affinity of fetal hemoglobin and a concentration differential across maternal and fetal vascular beds, allows for oxygen exchange via passive diffusion [2,6]. Facilitated diffusion and active transport mechanisms drive the exchange of carbohydrates, amino acids, lipids, vitamins and other nutrients required for fetal development [2,6]. Hydrostatic and osmotic pressures regulate fluid balance across the placenta and potassium, magnesium, calcium and phosphate ions are actively transported into fetal circulation [6]. The placenta is also an important endocrine organ. Several hormones secreted primarily by the syncytiotrophoblast (SCT) layer of the placenta are important for maintaining pregnancy and regulating processes necessary for gestation such as decidualization, implantation, placentation, angiogenesis and fetal growth [1,6]. Essential hormones include human chorionic gonadotropin (HCG), progesterone, oestrogens, human placental lactogen (HPL), human placental growth hormone (HPGH) and others [1]. Furthermore, the placenta serves as an immunoregulatory organ in two ways. It promotes
tolerance of the embryo by the maternal immune system through complex adaptations driven by trophoblasts, regulatory T cells and uterine natural killer (uNK) cells [3]. It is also involved in preparing the fetal immune system for life outside the womb. Beginning at approximately 16 weeks gestational age (GA), maternal antibodies are transferred across the placenta via receptor-mediated mechanisms, providing passive immunity to the developing baby for protection against pathogens likely to be encountered after birth [6,7]. Finally, through features such as export pumps and enzymatic metabolism, the placenta serves as a protective barrier against xenobiotic substances including drugs, carcinogens, environmental pollutants and hydrocarbons [6]. However, several drugs including alcohol, thalidomide, lithium and others cross the placental barrier and result in teratogenic effects on the fetus [6].

1.1.2 Classifications

There are several placenta types across eutherian mammals, classified by shape and degree of invasion into maternal tissues. Discoid describes the circular disk-shaped placenta seen in humans, primates and rodents [3]. Alternatively, the diffuse placentae of horses, pigs and whales show villi scattered across the majority of the chorionic surface [3]. Elephants and carnivores such as dogs, cats and bears have a placenta shaped as a band surrounding the fetus, which is described as zonary [3]. Finally, ruminants exhibit a cotyledonary placenta with multiple areas of attachment called placentomes [3,4].

In addition to morphological descriptions, eutherian placentae are classified into three major categories based on the number of cell layers between fetal and maternal blood. Horses, pigs and ruminants have an epitheliochorial placenta in which fetal and maternal blood are separated by several layers of epithelial and connective tissue [3]. Carnivores exhibit a more
invasive endotheliochorial placenta whereby conceptus-derived trophoblasts contact the endothelium of maternal blood vessels [3]. Finally, humans, apes and rodents have the most invasive placental type, called a hemochorial placenta. In hemochorial placentation, the endothelium of maternal arterioles is destroyed and the chorion is directly bathed in maternal blood [2–4,6]. This thesis will focus exclusively on the human placenta, which is classified as hemochorial and discoid [3,6] with a villous blood flow arrangement [2].

1.2 Trophoblast differentiation

1.2.1 Trophoblast lineage trajectories and anchoring column formation

Approximately 7-8 days after fertilization, the embryo implants in the uterine epithelium and the trophectoderm, the external layer of the blastocyst, invades to form the early placenta [8,9]. Trophoblasts, specialized placenta cells of epithelial lineage, form the maternal-fetal interface and, together with maternal stroma and immune cells, coordinate placenta development [10]. During early placentation, bi-potent progenitor cytotrophoblasts (CTB) from the trophectoderm differentiate down two distinct cell pathways: the villous and the invasive extravillous pathways [8–12]. The villous pathway forms the floating villi of the placenta, while the extravillous pathway drives the formation of anchoring villi that attach and invade the uterine epithelium [8,12,13] (Figure 1). Together, these two lineage trajectories give rise to the diverse trophoblast subtypes that form a functional placenta [10].

In the villous pathway, mitotically active CTB undergo cell-cell fusion events to form the SCT, a multinucleated outer cell layer of the chorionic villi [10,11,14] (Figure 1). The SCT is the major site of nutrient and gas exchange between maternal and fetal circulations, as well as the primary site of hormone production in the placenta [12]. Importantly, CTB fusion into SCT is
thought to be controlled by several mediators, but particularly the endogenous retroviral
glycoproteins syncytin-1 and syncytin-2 [15,16].

The differentiation pathway that results in the formation of invasive subtypes of
trophoblasts, and the focus of my thesis, is the extravillous pathway. The extravillous pathway
drives several key gestational processes including trophoblast column formation, column
trophoblast-mediated placental anchoring to the uterus, uterine spiral artery remodelling and
maternal immune cell tolerance towards the placenta and fetus. Differentiation along the
extravillous pathway and subsequent anchoring column formation occurs at points of contact
between placental villi and the uterine epithelium [10,12]. Proliferative CTB break through the
syncytium to form columns of trophoblasts, which are now called extravillous trophoblasts
(EVT), that establish an anchoring point of attachment between the placenta and the uterus
[10,12] (Figure 1). Column EVTs differentiate into highly invasive subtypes of EVTs called
interstitial EVTs (iEVT), which invade into the endometrial stroma, and into endovascular EVTs
eEVT), which migrate into maternal spiral arteries [10,12] (Figure 1). The iEVT and eEVT
contribute to spiral artery remodelling by destroying the musculature of the blood vessels and
displacing maternal endothelial cells, thus creating a low-resistance, high-perfusion blood flow
system [12,17]. Another subtype of extravillous trophoblast, called endoglandular trophoblasts,
are thought to invade uterine glands and open them towards the intervillous space, presumably to
promote histiotrophic nutrition [18,19]. An understanding of the villous and extravillous
differentiation pathways, anchoring column formation and the diverse trophoblast subtypes is
central to discussion of the effects of oxygen tension on EVT column outgrowth.
Along the villous pathway, CTB progenitors differentiate into the multinucleated SCT, forming the outer cell layer of the chorionic villi. Along the extravillous pathway, CTB progenitors migrate and differentiate into proximal column cytotrophoblasts (prox CCTB), distal column cytotrophoblasts (dist CCTB) and EVT, including iEVT and eEVT subtypes.
1.2.2 Factors controlling extravillous trophoblast differentiation

As trophoblasts differentiate along the villous and extravillous pathways, they undergo extensive changes in gene expression and adopt distinct cellular characteristics. These processes are controlled by an intrinsic genetic programming [20], but interactions with extracellular matrix (ECM), with maternal cells of the uterus, soluble factors produced by maternal cells, and changing oxygen gradients within the intervillous space are also thought to be involved [17]. For the purposes of this thesis, we will focus on the diverse molecular mechanisms controlling trophoblast differentiation along the extravillous pathway.

Firstly, cell-ECM interactions are fundamental to anchoring column formation, trophoblast migration and progression into the extravillous lineage [21,22]. Trophoblasts have specialized cell adhesion molecules (CAMs) called integrins that allow them to recognize and interact with their environment [23,24]. Integrins are heterodimeric proteins composed of α and β subunits involved in cell-cell and cell-ECM adhesion [25]. At least 18 previously identified α-subunits and 8 β-subunits produce approximately 23 distinct integrins with varying ligand specificities [25]. Integrins are known to be involved in key processes of placentation including trophoblast adhesion, migration and invasion, but the exact mechanisms regulating their expression and function are largely unknown [25]. During differentiation along the extravillous pathway, cytotrophoblasts lose the expression of integrin α6β4 (laminin receptor) and upregulate the expression of other integrins such as α5β1 (fibronectin receptor) [21,26,27] and α1β1 (collagen/laminin receptor) [28].

Growth factor-dependent protease systems like matrix metalloproteinases (MMPs) provide invasive EVTs with the ability to degrade decidual ECM [23,28]. There are 17 MMPs, but MMP-9 (gelatinase B) and MMP-2 (gelatinase A) have previously been shown to control
trophoblast cell line and primary CTB migration through Matrigel matrix (a basement membrane-like substance) [23,29]. Together, the MMPs can degrade nearly all components of the ECM including collagens, laminin, fibronectin, elastin, proteoglycans and gelatin [23]. MMPs are generally secreted as zymogens, which are activated in the extracellular compartment by their substrate proMMP-2 [23]. The ability of MMPs to degrade ECM is limited by tissue inhibitors of metalloproteinases (TIMPs), produced by the decidua and EVT in response to decidual transforming growth factor beta (TGF-β) [28,30,31]. Regulation of MMP activity by TIMPs has been proposed as a mechanism for controlling trophoblast invasion for successful placentation [31].

Another protease system with relevance to EVT differentiation is the a disintegrin and metalloproteinase (ADAM) family. ADAMs, along with MMPs, belong to the superfamily metzincins [30]. ADAMs are involved in trophoblast invasion through their ability to degrade ECM [30] and in regulating cell-cell and cell-ECM interactions by adhering to integrins [30,32]. ADAMs are composed of a pro-domain, a metalloproteinase domain, a disintegrin domain with a cysteine-rich region, an epidermal growth factor (EGF)-like repeat, a transmembrane domain and a cytoplasmic tail [10,32]. ADAMs are also implicated in activation of a variety of chemokines, cytokines and growth factors, such as EGF, through cell membrane-receptor interactions [30]. Furthermore, they are involved in ectodomain shedding of a variety of CAMs like vascular cell adhesion protein 1, N-cadherin and CD44 [30]. Finally, ADAMs are thought to contribute to the regulation of important cellular transduction pathways such as Notch- and Wnt-signalling [30]. Despite these known functions, the exact role of ADAMs in early placentation remains largely undefined [10]. Some studies have proposed involvement of ADAMs 10, 12 and 17 in preeclampsia, suggesting they may contribute to placental function [33–35]. With regards to
EVT differentiation, ADAM12 is the most well-characterized. ADAM12 exists as two splice variants resulting in a transmembrane and a secreted form, both of which exhibit proteolytic activity [30]. ADAM12-mediated shedding of the EGF receptor (EGFR) ligand, heparin binding epidermal growth factor, and the Notch ligand, delta like-1, is involved in signal transductions that regulate cellular differentiation, migration and proliferation [10]. Evidence suggests that this Notch-signalling pathway is critical in murine EVT-directed spiral artery remodeling [36]. Importantly, ADAM12 localizes to anchoring column EVTs in placental villi and invasive EVTs in Matrigel-embedded explants [37]. At least one, but potentially both splice variants of ADAM12 have been shown to promote the invasive EVT phenotype [10,37,38]. ADAM28 has also been shown to localize to human leukocyte antigen-G (HLA-G) positive trophoblasts and promote EVT column outgrowth [39]. Further investigation is required to elucidate potential involvement of ADAM12 and other ADAM family members in trophoblast differentiation [30].

Several cell signalling pathways, such as the aforementioned Notch pathway may play a role in EVT differentiation. The Notch receptor family (Notch1-4) is a group of single-pass transmembrane proteins with an extracellular domain and a Notch intracellular domain, which interact with membrane-anchored proteins [40,41]. Notch signal activation induces expression of a variety of genes involved in cell proliferation, including MMPs [40]. The Notch pathway is known to be important for placental development in mice [41]. Decreased Notch signalling has been observed in pregnancies affected by preeclampsia [42] and intrauterine growth restriction [43], suggesting that it may be involved in placentation. Notch receptors and ligands are expressed in various trophoblast subtypes [44], but the exact role of Notch in human trophoblast differentiation is poorly understood [40]. Notch1, 3 and 4 are expressed in CTB progenitors whereas Notch2 and Notch 3 are expressed in invasive EVTs [36,44]. There is some evidence to
suggest that Notch signalling blunts trophoblast motility and differentiation along the extravillous pathway [40]. Furthermore, a decrease in Notch activity has been observed during EVT differentiation in-vitro [45]. Together, this suggests a likely role for Notch signalling in stem cell maintenance and controlling rates of EVT column outgrowth [44].

Another signal transduction pathway thought to play a role in trophoblast differentiation and column outgrowth is Wnt signalling. Wnts are a family of cysteine-rich glycoproteins that are secreted through exosomes or bound to lipoproteins [46]. Through a series of intermediate steps, canonical Wnt signalling results in stabilization and nuclear translocation of de-phosphorylated β-catenin, which leads to activation of several transcription factors associated with growth and invasion-related genes [46]. In adults, Wnt controls tissue homeostasis of regenerating tissue and dysregulation of Wnt signalling is involved in several diseases including cancer and diabetes [46]. Wnt signalling is known to play an important role in stem cell maintenance, implantation, trophoblast differentiation, EVT motility and invasion [20,46–48]. A recent study by Haider et al., provides convincing evidence that endogenous Wnt signalling is a requirement for EVT development in human placental organoids [49]. Finally, the involvement of Wnt signalling in the etiology of preeclampsia and recurrent miscarriage further supports its potential role in placentation [20].

Paracrine stimulation through insulin-like growth factor 1, which is secreted by the villous mesenchyme, is involved in promoting trophoblast migration into the decidua and myometrium [22]. As trophoblasts migrate further away from the mesenchymal core, this signal diminishes, thus regulating depth of invasion [22]. Trophoblast invasion is also controlled by a variety of factors expressed by the maternal decidua. These include leukemia inhibitory factor, EGF and interleukin-11 [26], as well as activins and chemokines [28]. Because trophoblast
invasion is typically limited to the proximal third of the myometrium, the decidua is thought to restrict invasion [23,28]. Furthermore, EGF, vascular endothelial growth factor (VEGF) and various cytokines secreted by endometrial glands are involved in controlling placentation and early trophoblast differentiation events [28]. Finally, several transcription factors are implicated in column formation and trophoblast differentiation, but few of them have been functionally investigated [28].

The complex interplay between these diverse molecular regulatory mechanisms is ultimately what controls anchoring column formation and trophoblast differentiation down the villous and extravillous pathways. If these key developmental processes become dysregulated, it may lead to pregnancy complications.

1.3 Placental insufficiency and obstetrical complications

The highly invasive nature of the human placenta is critical to its function. Trophoblast invasion into the uterine stroma is necessary for interstitial implantation of the blastocyst into the endometrium [50]. CCTB differentiation into invasive EVT is a requirement of spiral artery remodelling. Recall that spiral artery remodelling involves infiltration of eEVTs into maternal arterioles, where they replace maternal endothelial cells [50,51]. Remodelling converts contractile maternal blood vessels into dilated, low-pressure conduits of blood flow, unresponsive to vasoconstriction [5,6,11,52,53]. This process allows for the direct contact between chorionic villi and maternal blood, which is the distinguishing feature of hemochorial placentation and is required to meet the aerobic and metabolic demands of the human fetus. Failure of early establishment of the extravillous pathway is a significant cause of obstetrical complications.
Poor EVT invasion into the uterine stroma leads to placental insufficiency, which is characterized by poorly remodelled spiral arteries and thus reduced blood flow, oxygenation and nutrient delivery to the fetus [2]. Impaired placentation is associated with many of the most pressing obstetrical complications including miscarriage [2,17], intrauterine growth restriction (IUGR) [2,17,52,53], placental abruption [52], premature rupture of the membranes [52], preeclampsia (PE) [2,52–54] and late gestation spontaneous abortion [52]. Importantly, several of these complications regularly result in preterm birth (PTB) [2]. PTB, which is defined as delivery before 37 weeks GA [55,56] is the leading cause of infant morbidity and mortality worldwide [56]. Consequences of PTB for the infant are severe and include increased risk of infection [57], respiratory failure [58] and physical and psychological disabilities such as cerebral palsy, developmental delays, epilepsy, blindness and hearing loss [59]. Other causes of PTB include several genetic and environmental factors, as well as intrauterine infections [2]. For the purposes of this thesis, I will focus my discussion of obstetrical complications on the two disorders of pregnancy most convincingly associated with placental insufficiency: PE and IUGR.

1.3.1 Placenta and preeclampsia

PE is defined by the onset of high blood pressure (>140/90 mmHg) detected on at least two occasions and proteinuria (>300 mg/l per 24 hrs) after 20 weeks GA [1,60]. PE affects 2-8% of pregnancies and is the leading cause of maternal morbidity and mortality [1,61,62], resulting in 10-15% of maternal deaths globally [63]. A subset of preeclamptic pregnancies are characterized by interstitial EVT invasion only as far as the decidua and eEVT remodelling of only the peripheral portion of spiral arteries [64]. This insufficient remodelling is problematic not only due to the decrease in blood volume delivered to the placenta (placenta ischemia), but also
due to an increase in blood pressure upon delivery [2,65]. Poor remodelling results in high-resistance vessels, causing increased sheer force of maternal blood flow into the intervillous space, resulting in significant damage to floating placental villi [2,64]. Furthermore, the vascular dysfunction associated with PE leads to intermittent malperfusion and subsequent oxidative stress [64]. Together, these mechanical and oxidative stressors lead to systemic inflammation and result in the maternal decompensation observed in PE [64]. Potential consequences of severe PE for the mother include eclampsia (seizures), blindness, cerebral hemorrhage [66], haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, and death [63]. Currently, the only effective treatment for PE is delivery of the fetus and placenta [66–69], frequently resulting in PTB. Given that PE is characterized by reduced blood flow to the placenta and thus failure to meet the aerobic and metabolic demands of the fetus, it often co-occurs with IUGR.

1.3.2 Placenta and intrauterine growth restriction

IUGR is defined as a critically low fetal weight for gestational age (<10th percentile) accompanied by aberrant intrauterine conditions [70,71] and it is the most frequent cause of death in the perinatal period [1]. Importantly, IUGR is a subset of small for gestational age (SGA), which describes babies that are born weighing below the 10th percentile with or without signs of aberrant intrauterine conditions. IUGR is associated with significantly more perinatal morbidity and mortality than SGA in general [1]. Potential causes of IUGR include genetic anomalies, maternal infections, metabolic syndromes and placental disorders [71,72]. IUGR is often accompanied by PE, but it can also arise without symptoms of PE (high blood pressure and proteinuria) [2]. As with PE, a subset of pregnancies affected by IUGR show reduced EVT migration and vascular remodelling, resulting in insufficient perfusion and nutrient transfer.
across the placenta [72]. Hypoxia, oxidative stress and ischemia/reperfusion events cause epithelial injury in chorionic villi, leading to dysregulation of placental functions [72]. IUGR is associated with several adverse perinatal outcomes including respiratory distress, necrotizing enterocolitis and neonatal death [73]. Moreover, IUGR and low birth weight is thought to cause an increased risk for metabolic and cardiovascular diseases [71,74] and neurodevelopmental disorders [75] in adulthood. Discussion of the most pressing obstetrical complications associated with placenta insufficiency is central to an appreciation for the importance of the invasive EVT differentiation pathway. As previously mentioned, this differentiation process is largely controlled by an intrinsic genetic programming. However, this programming can be adjusted by a variety of environmental factors including changes in oxygen conditions, hormones, cytokines and growth factors. One of the most significant, yet poorly understood factors affecting trophoblast differentiation is oxygen tension.

1.4 Oxygen and placentation

Early in gestation (8-10 weeks GA), trophoblasts aggregate and occlude the maternal spiral arteries, restricting the flow of oxygenated blood to the placenta [8,76–79] (Figure 2). For this reason, early placentation occurs in a hypoxic environment with an oxygen tension of 1-6% or approximately 10-30 mm Hg [2,8,65,80,81]. Later in the first trimester (after 12 weeks GA), trophoblast plugs dissipate, and maternal blood flow abruptly increases, creating an environment with oxygen conditions of approximately >6%, or 40-80 mmHg [8,65,78,81] (Figure 2). These ranges in oxygen saturation are the result of differences in the health of the pregnancy, as well as sampling sites in the intervillous space and sampling methods [82]. The sudden change in intrauterine oxygen tension at the end of the first trimester is thought to regulate trophoblast
differentiation into the villous and extravillous subtypes. However, the cellular and biological pathways that control this process are poorly understood.

Figure 2. Schematic diagram of trophoblast plugs and oxygen tension

Due to extravillous trophoblast plugs in maternal spiral arteries, oxygen concentrations in the intervillous space are comparatively low (1-6%) before 10 weeks of gestation. After 12 weeks of gestation, trophoblast plugs disappear, maternal blood flow increases, and oxygen concentrations rise (6-12%). This figure was adapted from Chang et al. [8].
Hypoxia is recognized as a critical feature of placenta development and function during the first trimester of pregnancy [8,76,80,83–85]. Low oxygen conditions cause changes in several signalling pathways that regulate gene expression, metabolic homeostasis and cell survival. Defects in these pathways can thus result in placental pathologies and contribute to obstetrical complications such as PE and IUGR. These pathways include hypoxia inducible factor (HIF), mammalian target of rapamycin (mTOR) and unfolded protein response [2,8]. HIF is the major molecular sensor of oxygen conditions in the placenta [2,65,83,86] and the focus of this discussion.

1.4.1 The role of hypoxia inducible factor

HIF is a helix-loop-helix protein composed of an alpha subunit (HIF-1α, HIF-2α or HIF-3α) and a beta subunit (HIF-1β) [83–85]. HIF-1 induces gene expression by binding to the N-terminal of the hypoxia response element on the promoter or enhancer region of several hundred genes [2,80,86]. Through this mechanism, HIF-1 regulates many cellular processes including angiogenesis, cell migration, invasion and metabolism, which are important for placentation [83]. Furthermore, a variety of research has demonstrated the importance of the HIF complex in healthy placenta development. For example, knockdown of HIF-1α and HIF-2α in transgenic mouse models has been shown to result in aberrant EVT differentiation and insufficient angiogenesis of the placenta [80,87]. An absence of HIF signalling leads to compromised formation of trophoblast giant cells (mouse equivalent of EVT) and exclusive differentiation of trophoblast stem cells into labyrinthine trophoblasts (mouse equivalent of CTB) [8,80,87,88]. Exposure of rats to low oxygen (11%) in early pregnancy has also been observed to promote/enhance the invasion of trophoblasts into the maternal blood vessels [8]. Finally, an
increase in HIF-1α expression has been implicated in CCTB to EVT transition in early gestational human placentae both in-vivo and in-vitro [85]. Despite these critical findings regarding the importance of HIF signalling in placentation, there are conflicting results about the role of oxygen tension in regulating trophoblast differentiation during the first trimester of pregnancy.

1.4.2 Debate about oxygen

Some studies suggest that low oxygen conditions drive trophoblast differentiation along the EVT pathway, while others claim the opposite effect. For example, research by Genbacev et al. and Caniggia et al. demonstrate that, when compared to culture in 20% oxygen, first trimester villous explants (5-8 weeks GA) cultured in low oxygen conditions show increased column expansion [89,90]. In contrast, James et al. suggest that villous explants cultured in 1.5% oxygen produce significantly less EVT column outgrowth when compared to those cultured in 8% oxygen [91]. Importantly, Chang et al. propose that much of the disagreement in the literature is due to a misunderstanding of terminology. Some discussions refer to human EVT as ‘intermediate’ trophoblast or ‘CCTB’ and suggest that hypoxia promotes CCTB proliferation. However, upon further examination, it becomes clear that these authors refer to an HLA-G+ cell lineage, suggesting EVT [8]. CCTB are defined as the trophoblasts immediately adjacent to the mesenchymal core of the chorionic villi. Trophoblasts that have invaded into the uterine stroma should be referred to as EVT [8]. It should be noted that CCTBs express EGF receptor (EGFR), whereas differentiated EVTs have lost the expression of EGFR and gained the expression of HLA-G [8]. This is the criteria I will use to distinguish between the two trophoblast populations for the purposes of my thesis. Another source of controversy over the effects of oxygen tension
on trophoblast differentiation stems from differences in study design. There exist a variety of models for studying human placentation with benefits and disadvantages to each. The choice of model is important because it can have a significant impact on research results.

1.5 Models for studying the placenta

The effect of oxygen tension on trophoblast differentiation has been an area of extensive research for ~20 years [8]. This early developmental process in human placentation is critical for establishing a healthy pregnancy. However, first trimester human pregnancy is exceedingly difficult to research for obvious ethical reasons. Fortunately, there are several in-vivo and in-vitro models that can provide some insight into the cellular and molecular mechanisms controlling early placentation.

1.5.1 Animal models

A significant amount of trophoblast research has been conducted on animal models, which are invaluable to the study of human reproduction for their multi-system, organism-level complexity [92]. However, animals have a wide variety of reproductive strategies and they differ in their similarity to humans. For this reason, there are several important considerations when selecting an animal model for the study of human reproduction. These include number of offspring per conception, placental classification [epitheliochorial, endotheliochorial or hemochorial], duration of pregnancy, and degree of neonatal development [92]. Small animals such as rodents and rabbits have a comparatively short gestational length and large litter size. This strategy offers several advantages for research, such as the ability to acquire a large amount of data over a short period of time and the feasibility of studying trans-generational effects [92].
In contrast, the longer gestation of guinea pigs, for example, can be beneficial for monitoring fetal development over time [92]. Consideration of placental morphology and degree of trophoblast invasion is crucial to the study of oxygen and nutrient transfer across the placenta. Rodents, rabbits, guinea pigs and great apes have a hemochorial placenta, which may make them more analogous to humans [92]. Trophoblast invasion is comparatively shallow in mice and rats, but deeper in guinea pigs. Unsurprisingly, non-human primates have deep trophoblast invasion and spiral artery remodelling, which most closely resembles that of the human [92]. Ultimately, the choice of animal model for a given study will depend on ethics, feasibility and research goals.

Rodents are used extensively in research and they offer several advantages for the study of human placentation. Rats and mice are widely available, inexpensive to house and breed, they allow for the manipulation of gene expression and provide a variety of similarities to the human placenta [93]. Both the mouse and the human have a hemochorial placenta, whereby the SCT is in direct contact with maternal blood [93–95]. The labyrinth layer of the mouse placenta is parallel in function [nutrient and gas exchange between maternal and fetal blood] to the chorionic villi of the human [94]. Previous research demonstrates several transcription factors involved in regulating placental gene expression, which are conserved between human and mouse [96,97]. Research by Cox et al. found >7000 orthologous genes with 70% co-expression between mouse and humans [98]. Furthermore, they found that >80% of genes known to regulate placenta phenotypes in mice are conserved in humans [98]. Despite these similarities, there are significant differences between the reproductive strategies of mice and humans that raise concerns about the extrapolation of results from mouse studies to applications for human health [99,100].
One anatomical difference between mouse and human is that the mouse placenta maintains three layers of trophoblasts separating fetal and maternal circulations, whereas the human has two layers in the first trimester of pregnancy and only one functional layer later in gestation [93]. Mice have a labyrinthine placental structure and a highly efficient countercurrent exchange system. Alternatively, humans have a villous placental structure whereby maternal blood is delivered in spurts and pools. The anchoring trophoblast cells of the mouse are significantly less invasive than those of the human [93]. Mice and humans also differ in the decidualization and implantation processes. Decidualization is a continuous, cyclical process of the human menstrual cycle, but it is induced by implantation of the embryo in the mouse [99].

Human gestation is 9 months, whereas mouse gestation is approximately 20 days. By 21 days GA, the human placenta has a definitive chorionic villus structure and trophoblast differentiation has already begun [99]. By comparison, the definitive structure of the mouse placenta is not observed until halfway through gestation and trophoblast invasion occurs much later [99]. Finally, mouse and human placental endocrine functions are vastly different with respect to the biological mechanisms controlling corpus luteum maintenance and progesterone production [99].

Despite these similarities and differences, mouse models are commonly used to study human reproduction. Over 100 mutant mouse models have been developed to express defects in placentation, and murine trophoblast stem cell lines have led to a better understanding of the mechanisms controlling stem cell maintenance and cell differentiation [101]. Finally, several mouse models have been uniquely developed to mimic PE [102–105] and IUGR [105,106].

Despite their many advantages and widespread availability in research settings, mice and rodents are still an imperfect model. Therefore, some investigators turn to non-human primates as a more closely related model for the study of human reproduction.
Old world monkeys, such as rhesus macaques and baboons are used to study human implantation, placentation and parturition, as well as reproductive pathologies such as endometriosis [100]. There are several characteristics of non-human primate models that make them relevant to the study of human reproduction: menstrual cycle similarities, villous placental morphologies, singleton pregnancies, similar endocrine regulation of pregnancy, similar process of decidualization and parallels in immune regulation [100,107]. For example, the rhesus macaque provides comparable expression of major histocompatibility complex class I genes, which are important for immune surveillance and thus have implications in fetal tolerance [107]. Furthermore, homologs of HLA-G receptors are expressed in rhesus decidual leukocytes [107]. Trophoblast invasion occurs rapidly after implantation in the rhesus macaque, as it does in the human [107]. Another advantage of the rhesus macaque is that there already exists a large amount of data and established laboratory reagents for use on the species [107]. However, there have been limitations regarding the availability of female rhesus macaques in recent years [107]. In addition to the rhesus macaque, the other commonly use primate for the study of human reproductive biology is the baboon.

The baboon reproductive system is comparable to that of the human with regards to menstrual cycle length, duration of pregnancy and oestrogen and progesterone secretions [108]. Advantages of the baboon include their larger size, which allows for greater tissue sampling and which more closely mirrors human physiology [107]. However, baboon trophoblast invasion is significantly less extensive than in the human, making the baboon an unreliable model for the study of placental insufficiency and PE [109]. Relative depth of trophoblast invasion is only observed in chimpanzees and gorillas, which are unlikely models for research due to their endangered status [100]. Although animal models are invaluable to the study of reproduction for
their multi-system complexity, they are limited in availability and applicability to human biology. Therefore, several ex-vivo and in-vitro techniques have been designed to model human systems.

1.5.2 Primary isolated trophoblasts

Culture of primary trophoblasts isolation from fresh placenta samples is a practical application of otherwise discarded human placentae that provides important insight into human placentation. There are several methodological variations of placental CTB isolation, but almost all protocols are modelled from Kliman et al. [110,111]. The standard method involves mincing of the tissue, followed by dissociation via enzymatic (trypsin, collagen or dispase) or chemical (EDTA) digestion [112,113]. Cells are typically passed through a 100-micron filter to remove cell aggregates and cellular waste, and purified using Percoll or Ficoll density gradient centrifugation [114]. A negative magnetic bead/antibody purification is often used to select for a specific trophoblast subtype [110,114]. Finally, cell purity is assessed by cytokeratin-7 expression via flow cytometry and in-vitro culture [110,115].

Despite the practicality of this model, there are several potential disadvantages of using isolated primary trophoblasts in reproductive biology research. For example, trypsin exposure tends to reduce cell survival and impact surface protein expression [110]. Moreover, there is disagreement in the literature regarding which biomarkers and isolation techniques provide the most consistent results [110]. It is difficult to determine from primary cultures the specific cell types responsible for various molecular functions, and reproducibility is a challenge given heterogeneity of primary samples [116]. Finally, isolated primary trophoblasts are limited in their ability to proliferate in culture. They survive for just a few days, allowing for short experiments.
only [49,112,117]. Given this limitation, there is a need for immortalized trophoblast cell lines for research applications.

1.5.3 Immortalized primary trophoblast and choriocarcinoma cell lines

Immortalized primary trophoblast (HTR-8) and choriocarcinoma (BeWo, JEG-3 and JAR) cell lines have been developed as trophoblast models for more extensive experimentation. HTR-8 is a first trimester trophoblast line that has been transfected with the Simian virus-40 [112,116,118] to allow for proliferation in culture. BeWo, JEG-3 and JAR cell lines were isolated from choriocarcinoma, an invasive trophoblastic cancer. The BeWo cell line was first established by Roland Patillo [119] in George Gey’s laboratory at Johns Hopkins University in 1966 [120]. These immortalized cell lines are differentially suitable for various research applications. Both HTR-8 and choriocarcinoma cell lines are HLA+ and are thus used to model EVT s [121]. Both HTR-8 and JEG3 cell types have been described as possessing progenitor-like characteristics based on NOTCH1 expression [118]. BeWo and JEG-3 are frequently used to model villous trophoblasts because they secrete placental hormones including HCG, HPL, progesterone and oestrogens [122,123]. Furthermore, BeWo cells undergo syncytialization [124] and demonstrate regulation of syncytin 1 and 2 [16]. Based on selective expression of cell adhesion molecules (i.e. integrins) and ECM interactions, HTR-8 cells have proven useful for functional studies of adhesion while JEG-3 and JAR cells are often used for functional studies of migration and invasion [125]. Despite the many advantages offered by immortalized cell lines, they also pose unique challenges. Most notably, the extensive survival of choriocarcinoma cell lines implies potential for genetic mutations between passages, potentially altering research
findings [112,116,126]. For this reason, the choice of cell line for a given research application should be made based on phenotype and validated with other models [126].

1.5.4 Pluripotent stem cells

One method that provides consistent access to human trophoblast cells while avoiding the issue of genetic mutations observed in immortalized and cancerous cell lines is the use of stem cells. Stem cells derived directly from the trophectoderm of an embryo at the blastocyst stage and induced pluripotent stem cells provide insight into early trophoblast differentiation events [117]. Given the ethical and logistical challenges of obtaining human embryos, induced pluripotent stem cells are the more feasible model [116] and the focus of this discussion. CDX2+/p63+ CTB stem-like cells can be generated from human pluripotent stem cells cultured in the presence of bone morphogenic protein 4 and absence of exogenous basic fibroblast growth factor [127–129]. With exposure to the appropriate hormones and biomarkers, these cells can be further differentiated into SCT and EVT-like cells [127]. While pluripotent stem cell lines allow for investigation of early trophoblast differentiation events that have previously been difficult to study [130], they also pose several challenges. Firstly, induced pluripotent stem cells are limited in their ability to maintain self-renewal in the undifferentiated, CTB-like state [127]. Furthermore, concerns have been raised regarding key differences between induced pluripotent stem cells and primary isolated trophoblasts. For example, microarray experiments suggest that EVT-like cells derived from induced pluripotent stem cells retain expression of a variety of markers of pluripotency including OCT4, CDX2, EOMES and HAND1, which primary trophoblasts do not [131]. Furthermore, EVT-like and SCT-like cells derived from induced
pluripotent stem cells have more closely-related gene expression profiles than do primary EVTs and SCT, suggesting differences in the differentiation of these cell types between models [131].

1.5.5 Organoids

The unique benefit of trophoblast organoids for the study of human placentation is that they combine the potential for self-renewal with the inclusion of varied cell types in a 3D in-vitro model [49]. Haider et al. recently developed self-renewing human trophoblast organoids from purified 6-7 week GA primary villous CCTBs, which they cultured in a cocktail of Matrigel matrix, growth factors and signalling inhibitors [49]. Importantly, EGF signalling, inhibition of TGF-β and Wnt pathway reinforcement were requirements for long-term expansion of these cultures [49]. The gene expression profile of CTB organoid cultures closely mirrors that of primary CTBs, including the expression of markers for stemness [49,132,133]. These organoids spontaneously undergo cell fusion to generate a SCT layer [49]. Turco et al. have also generated organoids by seeding enzymatically digested trophoblast cells from 6-9 weeks GA placenta into Matrigel drops and culturing them in a medium composed of EGF, FGF2, Wnt activator CHIR99021, TGF-β and SMAD inhibitors, A83-01 and R-spondin 1 [132]. They were able to establish consistent trophoblast organoids after two passages in 91% of placenta samples attempted, and a random sample of three cultures have grown for longer than one year [132]. The organoids self-arrange into a structure that closely resembles chorionic villi in-vivo. The basement membrane is in contact with the Matrigel, and CTB and multinucleated SCT are detected inside the spheroid by Ki67 and CD46 expression, respectively [132]. The organoids successfully model villous endocrine functions including secretion of HCG, KISS1 and CSH1 [132]. Finally, they also show differentiation into HLA-G+ EVTs that are able to invade through
Matrigel matrix [132]. Interestingly, self-renewing organoids of endometrial tissue from non-pregnant women have also been developed and can be frozen down and revived without loss of function or proliferative ability [133]. These organoids are transcriptionally similar to primary tissue and develop characteristics of the pregnant endometrium when exposed to pregnancy hormones like HCG [133]. Together, these recently developed trophoblast and decidual organoid models have the potential to provide profound insight into the physiology of early human pregnancy. However, while they are a useful model, organoids require complex regulation of the biochemical environment and are thus difficult to establish. For this reason, there exists a need for a less complicated or labour-intensive 3D culture system, such as placental explants.

1.5.6 Villous explant cultures

One effective ex-vivo model for the study of human reproduction that combines human tissue with multicellular complexity is the villous explant in culture. Villous explants are structurally intact human chorionic villi that have been transplanted to a matrix-coated culture plate. Explants provide preservation of CTB proliferation [92] and inclusion of varied placental cell types such as immune cells, blood cells, endothelial cells and mesenchymal stromal cells, which closely emulate the in-vivo condition [116]. Placental explants in culture are commonly used to study the maternal-fetal interface [92,134] and its processes including oxygen and nutrient exchange, metabolism and endocrine functions [135]. More specifically, explants are used to study CTB proliferation, EVT column formation, EVT differentiation events and the effects of growth factors, cytokines and oxygen conditions on these processes [135]. There are two major types of explant experiments: observation of the effects of various experimental conditions on tissue explanted from a healthy pregnancy and comparisons of function between
villi extracted from healthy pregnancies with those complicated by known obstetrical pathologies [92,112,135]. Several factors must be taken into consideration when working with explanted human placental villi. Firstly, explants must be cultured on an ECM gel in order to produce CCTB outgrowth. Miller et al. recommend Matrigel matrix for modelling early invasion processes [135]. Furthermore, placental tissue should be examined under a dissecting microscope and villous tips with CTB columns should be selected for best results [135]. Given its human origin, 3-D structure and multicellular complexity, placental explant cultures are arguably one of the best models for studying the impact of various environmental conditions, including oxygen tension, on early placentation.

1.6 Rationale and hypothesis

1.6.1 Rationale

Proper development of the placenta is one of the most important biological processes that affect the short and long-term health of the mother, fetus and newborn. Placentation during the first trimester is critical for establishing a healthy pregnancy and it represents one of the first opportunities for health promotion and clinical intervention. The invasive characteristics of trophoblasts are fundamental to the development of EVT columns, which function to establish the maternal-fetal interface. Trophoblasts are critically important for anchoring the fetus to the mother's uterus, for remodeling maternal vasculature to ensure proper oxygenation and nutrition to the fetus, and for regulating the mother's immune system during pregnancy. If any one of these molecular systems malfunctions, it can lead to pregnancy complications that may significantly impede maternal and fetal health.
In many ways, placental explants in culture closely mirror the physiological conditions of human pregnancy. However, there remains significant disagreement in the literature about the effects of oxygen tension on early developmental processes of placentation, even among explant studies. For example, Caniggia et al. used an explant model to show that culture in low oxygen (3%) causes an increase in column outgrowth, when compared to culture in high oxygen (20%) [90]. In contrast, James et al. show that explants cultured in low oxygen (1.5%) exhibit significantly less EVT column outgrowth than those cultured in 8% oxygen [91]. Furthermore, Genbacev et al. suggest that explants cultured in hypoxic conditions experience impeded CCTB differentiation along the invasive EVT pathway [89]. Importantly, recent data has emerged from a primary CTB model and global RNA profiling which suggests that hypoxia promotes expression of a pro-EVT phenotype [85]. Given the variability in results from explant studies along with convincing evidence in support of hypoxia as a driver of trophoblast differentiation, the impact of oxygen tension on early processes of placentation remains a promising yet unresolved question in reproductive biology.

Thus, my research uses a placental explant model to examine how varying levels of oxygen affect trophoblast differentiation and column outgrowth. I selected an explant model for its previously discussed advantages, as well as the opportunity it provides to investigate a scientific discipline fraught with disagreement. I chose three experimental conditions in order to evaluate the effects of hypoxic (1%), physiologically normal (5%) and aberrantly high (20%) first trimester oxygen conditions on EVT differentiation.

The overarching goal of this project is to uncover the mechanisms that regulate trophoblast differentiation along the EVT pathway in an effort to better understand the physiology of healthy pregnancy. Research of this kind may eventually contribute to the
development of diagnostic tools and early interventions for pregnancy disorders that serve to improve reproductive outcomes for women and infants.

1.6.2 Hypothesis

I tested the hypothesis that low oxygen conditions regulate placenta development by promoting column outgrowth and differentiation down the EVT pathway. This hypothesis was based on previously published literature and preliminary data. To fully examine the effects of oxygen on placental column formation, my specific aims are to:

1. Examine how low, physiologically normal and high (for first trimester) oxygen levels affect trophoblast column outgrowth.
2. Identify gene pathways affected by specific oxygen conditions in extravillous columns.
3. Test the importance of novel gene pathways in trophoblast column outgrowth.
CHAPTER 2. MATERIALS AND METHODS

2.1 Research ethics and tissue collection

First trimester placental tissue samples (5-8 weeks GA) were obtained from consenting women aged 19-35 undergoing elective pregnancy termination through the Comprehensive Abortion and Reproductive Education (CARE) program at the BC Women’s Hospital and Health Center in Vancouver, British Columbia. Patients were consented into my study during their pre-procedure counselling sessions. All participants were aware of the risks of participation and provided written, informed consent. Identifying patient information was removed from tissue samples. Ethics approval (H13-00640; inflammation and placentation) was obtained from the Ethics Board on the Use of Human Subjects through the University of British Columbia.

2.2 Placental villous explant cultures

Ex vivo placental villous cultures were established with 5-8 week GA placental villi from terminated pregnancies (N=8). Placental villi were dissected under a light microscope, washed in cold phosphate buffered saline (PBS) and embedded in Millicell® cell culture inserts (12mm, 0.4μm pores, EMD Millipore) coated in 200μL of Matrigel® matrix basement membrane (Corning) (Figure 3). Inserts were placed in a 24-well plate containing 400μL of DMEM/F12 (1:1) culture media, supplemented with 0.1% penicillin, streptomycin and antibiotic-antimycotic. Explant cultures were established overnight in a humidified 37°C tri-gas incubator set at 5% oxygen and 5% carbon dioxide (Figure 3).

Following 24 hours of culture, EVT columns (n=200) were imaged using a Nikon SMZ 7454T tri-ocular dissecting microscope equipped with a digital camera. These images were used as a reference point for column measurements. Columns were divided evenly into three
experimental conditions: 1%, 5% and 20% oxygen. Explants were cultured for an additional 48 hours in their respective oxygen conditions (Figure 3). Following 48 hours of culture, columns were imaged a second time. These images were compared to the 24-hour images as a record of column outgrowth over time.

**Figure 3. Schematic representation of experimental design and workflow for Aims 1 and 2**

First trimester placental villi were examined under a light microscope and embedded into Matrigel® matrix. All explant cultures were established in 5% oxygen for 24 hours before culture in either 1%, 5% or 20% oxygen for an additional 48 hours. Following the total 72 hours of culture, column outgrowth was quantified using ImageJ software. A separate cohort of placental explants were cultured for RNA extraction of column cells and gene expression microarray (Chapter 2.4).
2.2.1 Column outgrowth quantification

Explant column outgrowth measurements were taken from the 24 and 72-hour explant images using the ImageJ software. Column area was measured by tracing from the base of the column to the distal column, encompassing all invading EVTs (Figure 4). The difference in column area over time was calculated by dividing the 72-hour measurement by the 24-hour measurement. Finally, the difference in column outgrowth between the 24 and 72-hour columns was compared across oxygen conditions.

**Figure 4. Schematic diagram of methodology for explant outgrowth area measurements**

Placental villi, column and direction of invasion are shown in white. Column area is shown in yellow.
2.3 Immunofluorescence microscopy

Explants were fixed in 4% paraformaldehyde (PFA) for 24 hours, stored in 70% ethanol and paraffin embedded. Blocks were sectioned (6 μm) and two slides from each block were stained with hematoxylin and eosin according to standard protocol. Tissue underwent antigen retrieval by heating in a microwave for 2 minutes, followed by another 2 minutes at 15-second intervals, in a sodium citrate buffer (pH 6.0). Following antigen retrieval, sections were incubated with freshly prepared sodium borohydride for 5 minutes at room temperature (RT) and permeabilized with 0.2% Triton X-100 for 5 minutes at RT. Tissue blocking was performed with 5% regular goat serum and saponin in PBS for one hour at RT. Sections were then incubated overnight at 4°C with one or more of the following primary antibodies: anti-HLA-G (1:100, 5A6G7, Thermo Fisher Scientific); anti-cytokeratin 7, mouse monoclonal IgG (1:50, C2206, Santa Cruz Biotechnology); anti-cytokeratin 7, rabbit monoclonal IgG (1:50, CS-8421, Ventana Medical Systems); anti-Ki67 (1:75, RM-9106-S1, Thermo Fisher Scientific); anti-LOX (1:100, NB100-2527, NovusBio); anti-PEG10 (1:100, 4C10A7, NovusBio); Bu20a (1:1000, 52925, Cell Signalling Technology). Following incubation with primary antibodies, sections were washed with PBS and incubated with Alexa Fluor™ goat anti rabbit-488/-568 or goat anti mouse-488/-568 conjugated secondary antibodies (Life Technologies) for one hour at RT. Glass coverslips were mounted onto slides with ProLong Gold anti-fade reagent containing 4’,6-Diamidino-2-phenylindole (DAPI) (Life Technologies). Slides were imaged using the Zen 2 Core Imaging software for Zeiss microscopes.
2.4 Differential gene expression analysis

Concurrently with the explants cultured for column outgrowth measurements and immunofluorescence microscopy analyses, an additional cohort of explants (n=15) from five new first trimester placentae (5-8 weeks GA) were cultured in the same experimental conditions for RNA extraction and gene microarray analysis (Figure 3). Explants were established in 5% oxygen for 24 hours and then cultured in either 1%, 5% or 20% oxygen for an additional 48 hours following the same experimental methods discussed above.

2.4.1 RNA extraction

Following 48 hours of culture in 1%, 5% or 20% oxygen conditions, villous tips were quickly microdissected from Matrigel® embedded EVT columns (Figure 5). Total RNA extracted from column and invasive EVT cells was prepared using TRIzol LS reagent (Ambion) followed by RNeasy MinElute Cleanup (Qiagen) and DNase treatment (Ambion), according to the manufacturer’s instructions. RNA purity was assessed using NanoDrop Spectrophotometer (Thermo Fisher Scientific) and 2100 Bioanalyzer Instrument (Agilent). All RNA samples had an RNA integrity number (RIN) > 8.0 and were used for gene expression analyses. Total RNA samples were sent to Génome Québec Innovation Centre at McGill University in Montréal, Québec for microarray hybridization.
Figure 5. Schematic diagram of microdissection of explant for gene expression array

Villous tips were discarded, and RNA was extracted from remaining CCTB and EVT for gene expression analysis.
2.4.2 Microarray analysis

Upon arrival at Génome Québec, column and EVT RNA samples were prepared for analysis using the GeneChip™ Pico Reagent Kit (Thermo Fisher Scientific). This preparation method allows for profiling of subpopulations of cells with small quantities of total RNA. Expression profiles of >20,000 well-annotated genes were measured via the Affymetrix Human Clariom™ S Array, which is a transcriptome-wide gene-level expression profiling tool that analyzes expression of key genes and gene pathways [136]. I used the Bioconductor oligo package [137] to read raw array data into RStudio software for statistical computing and graphics (version 1.0.143) and convert Affymetrix CEL files into an expression matrix of intensity values.

2.4.3 Data pre-processing

Data pre-processing was conducted in Vancouver and included background correction and normalization using the Oligo package for Bioconductor, as well as “soft” intensity-based probe filtering using the Limma package. Boxplots of probe intensity values for raw and cleaned data demonstrate that data pre-processing was successful at normalizing mean expression values across samples (Figure 6). A total of 13,787 control, duplicated, non-annotated or low intensity probes were filtered out of the data. This left 13,402 probes for further analysis. Preprocessing was monitored at each step by Principal Component Analysis (PCA) and linear modelling. Batch correction was omitted from pre-processing because the variable of interest (oxygen tension) was strongly associated with principal component 1 prior to correction (Figure 7). Furthermore, batch correction introduced a false positive effect of oxygen, which was observed through linear modelling. PCA of pre-processed data suggest that principal component 1 accounted for ~35% of the variance in the data.
Figure 6. Probe intensities from raw and pre-processed gene expression data

Boxplots representing the distribution of probe intensity values for each sample before and after normalization and filtering.
Figure 7. Principal Component Analysis (PCA) of normalized and filtered data

Principal component (PC) values (x-axis) are correlated with phenotypic data (y-axis) and experimental conditions according to coloured boxes. P-values are represented by blue shaded boxes as indicated by the legend. Oxygen exposure was strongly associated with principal component 1, which accounted for ~35% of the variability in the data.
2.4.4 Global differential gene expression

Comma separated values (CSV) files were generated for all differentially expressed genes, as well as differentially upregulated and differentially downregulated genes specifically, in comparisons between 1% and 20% oxygen cultured samples and 5% and 20% oxygen cultured samples. Global, unsupervised gene expression differences between samples cultured in the three experimental conditions were observed by applying global expression sets and CSV comparison files to volcano plots, PCA analyses and hierarchical clustering analyses. Volcano plots were generated using the ggplot2 package for RStudio. Cluster analysis of sample relations based on principal components was generated using the “plotSampleRelation” function for the Lumi package. A hierarchical cluster analysis was conducted using a Euclidian cluster algorithm of the top 40 differentially expressed genes, selected by false discovery rate (FDR) <0.05 and ranked by fold change.

2.4.5 Pathway analyses

Pathway analyses were conducted by applying various expression signatures to the ClusterProfiler package for Bioconductor. The ClusterProfiler software systematically incorporates annotated genes for various biological processes, molecular functions and cellular components from Gene Ontology (GO). Hierarchical cluster analyses were done on genes involved in specific pathways and biological processes, curated from the ToppGene portal for gene list enrichment. Hypoxia specific genes were amalgamated from GO terms “response to oxygen levels” (GO:0070482), “response to decreased oxygen levels” (GO:0036293) and “response to hypoxia” (GO:0001666). Genes involved in regulation of the cell cycle were derived from the GO term “regulation of cell cycle process” (GO:0010564). Growth factor and
cytokine binding genes were curated from GO terms “growth factor binding” (GO:0019838) and “cytokine binding” (GO:0019955). Finally, ECM-related genes were amalgamated from GO terms “ECM organization” (GO:0030198), “extracellular structure organization” (GO: 0043062) and “genes encoding enzymes and their regulators involved in remodelling of the ECM” (GO:M3468).

2.4.6 Targeted gene expression analyses

For hierarchical clustering of samples based on expression of trophoblast genes, I curated a list of previously identified genes from several peer-reviewed sources that are known to be associated with various stages of differentiation along both the villous and extravillous pathways [8,49,132,138–141]. I applied this list of genes to the expression signatures using a Euclidian gene-clustering algorithm to generate a heatmap of gene expression with sample and gene clustering. Furthermore, I identified the top 15 differentially upregulated genes from each of the 1% and 20% enriched signatures by fold change and FDR < 0.05. Victor Yuan examined the expression of these genes in publicly available single cell RNA-sequencing data generated from first trimester placentae, published by Vento-Tormo et al. [141]. Victor performed a pseudotime analysis using the Monocle package for Bioconductor. Finally, this signature was cross-referenced with known genetic markers of trophoblast subtypes in various stages of differentiation along the villous and extravillous pathways.
2.5 BrdU pulse-chase experiment

Pulse-chase labelling with bromodeoxyuridine (BrdU) was conducted on a verification cohort of Matrigel embedded placental explants derived from distinct 5-8 week GA placentae (n=2). Explants were established in 5% oxygen for 24 hours followed by 24 hours of culture in either 1%, 5% or 20% oxygen. After 48 hours of culture, explants were exposed to a 4-hour pulse with culture media containing 10μM of BrdU. Following 4 hours of labelling, explants were washed in PBS and fixed in 4% PFA overnight. Explants were paraffin embedded and sectioned for immunofluorescence microscopy. Immunofluorescent staining with anti-BrdU antibody (Bu20a) was conducted as previously described, with the addition of a 30-minute incubation in 2M hydrochloric acid between permeabilization and sodium borohydride steps. Imaging and image analyses were conducted with the Zen 2 Core Imaging software for Zeiss microscopes. BrdU expression was quantified as the ratio of BrdU+ cells to all nuclei in the proximal column.

2.6 LOX activity assay and LOX inhibition

Measurement of endogenous Lysyl Oxidase (LOX) activity in placental explant conditioned media (CM) was performed following a modified protocol described by Wiel et al. [142]. Briefly, 600 μl of CM from placental explants cultured in triplicate from either 1%, 5%, or 20% oxygen conditions was pooled, concentrated 12-fold using 7.5 kDa exclusion Amicon Millipore concentration columns (Millipore), and snap-frozen in liquid nitrogen. LOX activity in 15 μL of concentrated CM was determined using the Amplex Red H2O2 detection kit following the manufacturer’s instructions (Life Technologies). This assay is based on the ability of endogenous LOX to oxidize 10 mM of substrate 1,5-diaminopentane (DAP) in the presence of
0.5 U/mL horseradish peroxidase; the reaction is incubated at 37°C for 30 minutes. Oxidation of Amplex Red generates a fluorescent signal measurable at 560 nm/590 nm excitation/emission wavelengths that was detected with a fluorescence plate reader (BMG Labtech) using a 96-well plate format. As a positive control, 10 µg/mL recombinant active LOX (MyBiosource.com) was separately incubated with DAP substrate. LOX activity/specificity was determined by co-incubating reactions with 5 mM of β-aminopropionitrile (BAPN). For endogenous inhibition of LOX within explant cultures, following 24 hours of explant establishment at 37°C in a humidified tri-gas incubator, explant media was replaced with media containing 500 µM of BAPN. Explants were then cultured in either 1% or 20% oxygen for an additional 48 hours prior to imaging and quantification.

2.7 Statistical analysis

For parametric analyses, group means were compared via analysis of variance (ANOVA) with a Tukey multiple comparisons test. For non-parametric analyses (explant column outgrowth), group means were compared via a Kruskal-Wallis ANOVA with a Dunn’s multiple comparison’s test. Statistical significance was determined as P<0.05 and analyses were conducted with Prism7 software (GraphPad). Please refer to section 2.4 for statistical methods performed on the gene microarray analysis.
CHAPTER 3. COLUMN OUTGROWTH

3.1 Introduction

Development of the EVT column is required for anchoring of the placenta to the mother’s uterus and for trophoblast-directed vascular remodelling of uterine arteries [37]. Insufficient trophoblast invasion is associated with a number of undesirable pregnancy outcomes including spontaneous abortion, IUGR, premature rupture of the membranes and fetal death [52]. However, the biological mechanisms driving trophoblast column formation are poorly understood. Oxygen tension is one potentially important factor regulating trophoblast invasion and differentiation, but the evidence is conflicting [143]. Some studies have found that first trimester villous explants cultured in low oxygen show significantly reduced outgrowth when compared to higher oxygen conditions [91] and others have found the opposite effect [89], or no significant difference [76,144].

For ethical and logistical reasons, early human placental development is a challenging area of research. However, 3-D culture systems such as the villous explant model may be an effective method for studying trophoblast column formation in the first trimester of pregnancy. In this chapter, I aim to test the effects of varying oxygen conditions on EVT column outgrowth using placental explants cultured in either 1%, 5% or 20% oxygen.
3.2 Results

3.2.1 Efficacy of explant model confirmed through immunofluorescence microscopy

To investigate the effects of varying oxygen conditions on trophoblast differentiation along the invasive EVT pathway and anchoring column outgrowth, Matrigel-embedded villous explants derived from first trimester placental tissues (5-8 weeks GA) were cultured in 5% oxygen for 24 hours, followed by culture in either 1%, 5% or 20% oxygen for 48 hours. In-vivo anchoring column formation involves differentiation of CCTBs into highly invasive EVTs, which migrate through the uterine stroma and contribute to maternal spiral artery remodelling (Figure 8A). To this end, Matrigel embedded explants mirror aspects of trophoblast differentiation and migration along the EVT pathway and allow for observation of anchoring column outgrowth outside the human body (Figure 8B). This explant model allowed for reproducible and accurate imaging and column outgrowth quantification (Figure 8C).
Figure 8. Schematic diagram of EVT column formation in-vivo and in explant model

(A) Schematic diagram of trophoblast differentiation along the extravillous pathway and anchoring column formation. (B) Schematic diagram of EVT column formation in a Matrigel embedded explant model. (C) Representative image of EVT column outgrowth in an explant model.
To confirm that Matrigel-embedded placental explants reproduce similar developmental and differentiation events to in-vivo placental columns, I compared the expression and localization of pan-trophoblast cytokeratin-7 (Ck7), distal column cell/EVT HLA-G and proliferative MKI67 (Ki67) markers via immunofluorescence microscopy (Figure 9). Placental explants showed many similarities to in-vivo trophoblast columns in aspects of anchoring column structure and organization (Figure 9A, B). Specifically, Matrigel explants formed multi-cellular columns defined by both proximal and distal Ck7+ trophoblasts; proximal CCTB showed intense positivity for the proliferative antigen marker Ki67, whereas distal CCTB showed a gradual increase in expression of the EVT marker HLA-G (Figure 9C, D). As HLA-G+ cells detach from column structures and begin invading through the Matrigel matrix, they no longer stain positive for Ki67 and they show a reduced tendency for cell-cell adhesion, as observed through an increased proportion of isolated cells (Figure 9C). These cellular characteristics are conserved during EVT differentiation in-vivo. Overall, my explant model closely recapitulates many of the cellular and developmental events characterized in normal first trimester placentation.
Figure 9. Validation of the explant model via immunohistochemistry

(A) First trimester placenta in-vivo stained with proliferation marker Ki67 (pink) and EVT marker HLA-G (green). (B) In-vivo decidua section stained with HLA-G, illustrating column cells and EVTs (brown) that have invaded into the maternal decidua. (C) Histologically sectioned placental explant stained with Ck7 (white) and Ki67 (pink). Proliferative Ki67+ cells are localized to prox CCTB. (D) Histologically sectioned placental explant stained with Ck7 (white) and HLA-G (green), representing invasive EVTs.
3.2.2 Low oxygen promotes column outgrowth

Placental explants were established in 5% oxygen overnight and cultured in either 1%, 5% or 20% oxygen for 48 hours. In all conditions, column outgrowth was observed (Figure 10). Visually, the three different experimental conditions led to notable differences in column morphology. Culture in 1% oxygen led to columns featuring elongated protrusions (“spikes”) of outgrowth, whereas culture in 20% oxygen led to columns comprised of densely-packed cells (Figure 10). Notably, column outgrowth area was greater in the 1% and 5% oxygen conditions when compared to the 20% oxygen condition (Figure 11A). These observations suggest that distinct cellular processes in low and high oxygen conditions differentially contribute to column outgrowth. The effect of oxygen tension on column outgrowth is in part regulated by patient ID (Figure 11B). However, most patients generally follow the same trend with higher column outgrowth in the 1% oxygen condition and decreasing outgrowth with increasing oxygen tension (Figure 11B). Further, morphological changes indicate that the lower two oxygen conditions (1% and 5%) may promote column outgrowth by potentiating pro-migratory or pro-invasive processes, as indicated by the elongated protrusions of outgrowth observed in the 1% oxygen cultures (Figure 10). Alternatively, elevated oxygen (20%) may promote column outgrowth through processes less dependent on cell migration/invasion.
Figure 10. Column outgrowth over time and general column morphology

Representative images of column outgrowth between 24 and 72 hours of culture in 1%, 5% and 20% oxygen conditions. Traced outlines of column shape highlight morphological differences between columns cultured in the three experimental conditions.
Figure 11. Column outgrowth Area in 1%, 5% and 20% oxygen conditions

(A) Difference in column (n=200) outgrowth area between 24 and 48 hours of culture in 1%, 5% and 20% oxygen conditions (N=8 patients, 63 explants). Results of a non-parametric Kruskal-Wallis test with a Dunn’s multiple comparisons analysis show a significant difference in column outgrowth area between 1% and 20% oxygen conditions (P=<0.0001) and 5% and 20% oxygen conditions (P=0.0003). There is no significant difference in outgrowth area between the 1% and 5% oxygen cultured columns. Bars represent 10th and 90th percentiles. (B) Mean difference in column outgrowth area by patient ID. ANOVA suggests a significant difference in mean outgrowth between 1% and 20% (P=0.0095) and 5% and 20% (P=0.018) oxygen cultured samples. Patient ID is indicated according to legend.
3.3 Conclusion

In this chapter, I examined the effects of 1%, 5% and 20% oxygen conditions on EVT column outgrowth. I demonstrate that placental explant cultures are an effective model for studying trophoblast differentiation along the extravillous pathway and anchoring column outgrowth. I observed column outgrowth in all experimental conditions. Culture in 1% or 5% oxygen led to a statistically significant increase in column area when compared to culture in 20% oxygen. I observed clear differences in column morphology between conditions with the 1% oxygen condition showing more elongated protrusions of outgrowth in the distal column and the 20% oxygen condition showing dense plumes of outgrowth in the proximal column. These oxygen-dependent changes in column area and morphology may suggest that low oxygen tension promotes trophoblast differentiation along the extravillous pathway, driving column migration and invasion. These findings help to clarify some of the disagreement in the literature and improve our understanding of how oxygen tension regulates column outgrowth.
CHAPTER 4. GENE EXPRESSION ANALYSIS

4.1 Introduction

Healthy placentation involves the formation of anchoring columns, trophoblast differentiation down the extravillous pathway, and EVT invasion into the uterine stroma. These early developmental processes occur in low oxygen, before the onset of maternal blood flow into the placenta [8,145]. Oxygen tension is known to affect a variety of cell signalling pathways including HIF, mTOR, and others, which are involved in regulating trophoblast biology and contribute to the pathophysiology of several obstetrical complications [8]. There is significant controversy in the literature regarding the role of oxygen tension in controlling trophoblast differentiation. The specific biological mechanisms that regulate differentiation down the EVT pathway and anchoring column formation are unknown. To my knowledge, no previous studies have been conducted that combine an explant model with non-biased genomic approaches to examine the effects of oxygen tension on column outgrowth.

In the previous chapter, I showed that placental explants cultured in low (1%) or moderate (5%) oxygen demonstrate greater column outgrowth than explants cultured in high (20%) oxygen. Moreover, columns cultured in 1% oxygen appeared “spikier” and more invasive, whereas columns cultured in 20% oxygen appeared homogenous and dense. These initial observations suggest that low, moderate, and high levels of oxygen control trophoblast column outgrowth and EVT differentiation through distinct molecular mechanisms. The goal of this chapter is to identify specific molecular processes impacted by oxygen conditions during column outgrowth and trophoblast differentiation along the EVT pathway. Gene expression profiles are expected to reflect the significant changes in column outgrowth observed between samples cultured in various oxygen conditions.
4.2 Results

4.2.1 Explants cultured in low, moderate, and high oxygen conditions generate unique transcriptomic signatures

Explants were established and cultured from a new cohort of 5-8 week GA placentae (N=5) as previously described. Following standard probe filtering and normalization, differential gene expression comparisons (FDR <0.05) and PCA were performed between 1%, 5%, and 20% oxygen conditions. Differential gene expression analyses identified 293 upregulated and 685 downregulated genes when comparing the 1% oxygen cultured column trophoblasts to the 20% oxygen cultured column trophoblasts (Figure 12A). Similarly, 363 upregulated and 406 downregulated genes were identified when comparing the 5% oxygen cultured samples to the 20% oxygen cultured samples (Figure 12B). However, no differentially expressed genes were identified when comparing the 1% and 5% oxygen exposed cultures (Figure 12C).
Figure 12. Volcano plots of differentially expressed genes

Each point represents a gene. Points are plotted based on log fold change and adjusted P-value. FDR thresholds of <0.05 and <0.10 are indicated with horizontal lines. Orange points represent upregulated genes with an FDR <0.05 and blue points represent downregulated genes with an FDR <0.05. The total number of upregulated and downregulated genes are indicated by the orange and blue numbers, respectively. (A) Upregulated and downregulated genes in the 1% oxygen condition compared to the 20% oxygen condition. (B) Upregulated and downregulated genes in the 5% oxygen condition compared to the 20% oxygen condition. (C) No differentially regulated genes were identified when comparing the 1% and 5% oxygen conditions.
Cluster analysis based on principal components suggests that samples generally cluster according to oxygen condition with a single 1% oxygen sample clustered alongside the 20% oxygen specimens (Figure 13). Explants cultured in 5% oxygen cluster more tightly than those cultured in 1% or 20% oxygen. Overall, sample clustering demonstrates more within group variation than between group variation. Importantly, global gene-informed hierarchical clustering of explant samples segregated column trophoblast cultures into two major arms: those exposed to comparatively lower oxygen (1% or 5%) and those exposed to high oxygen (20%) (Figure 14). This clustering profile further illustrates the significant effect of exposure to 20% oxygen when compared to 1% or 5% oxygen and the lack of a significant difference in gene expression when comparing the two lower oxygen condition groups.

In both 1% and 5% oxygen conditions, the most highly upregulated genes identified by global differential gene expression analysis include those associated with hypoxia/low oxygen (EGLN3, RORA) and cell-matrix interactions (LOX, JAM2, EGLN3). Notably, highly expressed genes within the 20% oxygen cultured explants were almost exclusively related to cell division and cell cycle processes (MKI67, KIF20A, KIF23, NCAPG, CDC7, CDK1, NCAPH, TOP2A etc.), indicating that column trophoblasts cultured in 20% oxygen likely harbour a proliferative phenotype (Figure 14).
Figure 13. Hierarchical clustering based on principal components

Estimate of sample relations based on expression of 11,433 probes, selected for large coefficient of variance (SD/mean >0.1). This plot was generated using the “plotSampleRelation” function from the Lumi package for R. Principal component 1 accounted for 34.8% and principal component 2 accounted for 15% of the variation in the data. Each point represents one explant and points are colour-coded based on experimental condition, as indicated in the figure legend.
Figure 14. Unsupervised hierarchical clustering analysis

Heatmap illustrating sample clustering and expression of the top 40 genes, selected by FDR <0.05 and ranked by fold change. This plot was generated using a Euclidean cluster algorithm. Colour coded bars on top of the heatmap indicate experimental group (blue = 1% oxygen; purple = 5% oxygen; red = 20% oxygen) and patient ID (from black to white: P637, P638, P640, P647 and P654). Patient numbers and oxygen conditions are also listed below the heatmap.
4.2.2 Low oxygen promotes expression of EVT-related genes and high oxygen drives proliferation

To further elucidate the cause of increased column outgrowth in low oxygen conditions and identify the biological mechanisms involved in trophoblast differentiation along the EVT pathway, I turned to pathway analyses via GO annotations using the Oligo package for Bioconductor. Interestingly, culture in both the 1% and 5% oxygen conditions led to enrichment primarily of biological pathways involved in response to hypoxia, ECM structure and organization, and response to steroid hormones (Figure 15A, B). Alternatively, culture in 20% oxygen led to almost exclusive enrichment of biological pathways involved in promotion of the cell cycle including mitotic nuclear division, sister chromosome segregation, and DNA packaging (Figure 15C). These results support my observation that low oxygen conditions promote column outgrowth through a cell migration and invasion-rich phenotype while the high oxygen condition promotes column outgrowth through a more proliferative phenotype.
Figure 15. Gene pathways upregulated in low and high oxygen conditions

(A) ClusterProfiler dotplot of gene pathways upregulated in 1% oxygen when compared to 20% oxygen. (B) ClusterProfiler dotplot of gene pathways upregulated in 5% oxygen when compared to 20% oxygen. (C) ClusterProfiler dotplot of gene pathways upregulated in 20% oxygen when compared to 1% oxygen. The number of significant genes involved in the pathway is illustrated by the size, and adjusted P-value is represented by the colour of the dots, as indicated in the figure legend.
Further analyses were done to reveal the specific genes involved in pathways of interest. Using the ToppGene [146] portal for gene set enrichment analysis, I curated lists of genes involved in pathways of potential biological relevance including hypoxia related pathways, regulation of the cell cycle, growth factor and cytokine binding, and genes involved in ECM interactions (Figure 16). As expected, hypoxia related pathways were enriched in the low oxygen conditions (Figure 16A). Interestingly, genes involved in regulation of the cell cycle demonstrate a gradual increase in expression from the 1% oxygen condition to the 20% oxygen condition (Figure 16B). This observation supports my finding that exposure to high oxygen conditions promotes a proliferative phenotype in the EVT column. Similarly, growth factor and cytokine binding were shown to be upregulated in the lower oxygen conditions, suggesting that these processes may be involved in the invasive phenotype observed in the 1% and 5% oxygen cultured columns (Figure 16C). Furthermore, the expression of genes involved in regulating structure and organization of the ECM was upregulated in the low oxygen conditions (Figure 16D). As ECM degradation and remodelling is required for trophoblast migration and invasion into the uterine stroma, this result further supports my finding that low oxygen conditions promote column formation through a pro-EVT phenotype.
Figure 16. Heatmaps for biological pathways of interest

(A) Hypoxia specific heatmap curated from GO terms “response to oxygen levels” (GO:0070482), “response to decreased oxygen levels” (GO:0036293) and “response to hypoxia” (GO:0001666). (B) Cell cycle regulation heatmap curated from GO term “regulation of cell cycle process” (GO:0010564). (C) Growth factor and cytokine binding genes curated from GO terms.
“growth factor binding” (GO:0019838) and “cytokine binding” (GO:0019955). (D) Heatmap of ECM related genes curated from GO terms “ECM organization” (GO:0030198), “extracellular structure organization” (GO: 0043062) and “genes encoding enzymes and their regulators involved in the remodelling of the ECM” (GO:M3468).
To test whether the proliferative signature observed in the 20% cultured explants translates to a proliferative phenotype in column trophoblasts, I performed a BrdU pulse-chase proliferation analysis on a separate cohort of placental explants derived from distinct placenta samples (N=2) and cultured in 1%, 5% or 20% oxygen conditions. I hypothesized that if the 20% oxygen condition was indeed promoting the cell cycle, I would see more BrdU incorporation in the explants exposed to 20% oxygen. Consistent with the microarray data, little to no evidence of cell proliferation was observed following a 4-hr chase in explant columns cultured in 1% oxygen (Figure 17A). However, explants cultured in 5% oxygen showed a significant increase in BrdU+ column trophoblasts when compared to explants cultured in 1% oxygen, and this increase in proliferation was more pronounced in explants cultured in 20% oxygen (Figure 17A, B). While a trend for more proliferation in explant columns cultured in 20% oxygen compared to 5% oxygen was observed, this difference was not significant (Figure 17B). Overall, my findings suggest that explant column trophoblasts exposed to a hyper-physiological 20% oxygen condition adopt a predominantly pro-proliferative phenotype.
Figure 17. Columns cultured in 20% oxygen show increased BrdU incorporation

(A) Sections from explants cultured in 1%, 5% and 20% oxygen conditions, subjected to a 4-hour BrdU pulse-chase and stained with Bu20a (green, white) and Ck7 (pink). (B) Degree of BrdU incorporation was calculated as a percentage of BrdU+ cells to the total number of nuclei in the column. One-way ANOVA with a Tukey multiple comparisons test yields a statistically significant difference in BrdU incorporation between 1% and 5% oxygen conditions and 1% and 20% oxygen conditions. Each point represents a column and bars represent median with interquartile ranges.
4.2.3 Examining gene expression differences between 1% and 5% oxygen

Though global gene expression analyses did not reveal significant differences in gene expression between the 1% and 5% oxygen conditions, I was interested to examine the differences between comparisons of each of the lower oxygen conditions with 20% oxygen. A total of 53 genes were found to be upregulated and a total of 357 genes were found to be downregulated in the 1% oxygen condition exclusively, when compared to the 20% condition. Similarly, 123 genes were found to be upregulated and 78 genes were found to be downregulated in the 5% oxygen condition exclusively, when compared to the 20% oxygen condition. These results suggest that culture in hypoxic and normoxic conditions lead to subtly different gene expression signatures (Figure 18). GO pathway analysis of these genes via the ClusterProfiler package indicate that column trophoblasts exposed to 1% oxygen show enrichment of biological processes favoring hypoxia-related and oxidative stress signalling (Figure 19A). In contrast, column trophoblasts cultured in 5% oxygen showed enrichment of biological pathways linked to nucleotide biosynthesis and metabolism. These results suggest that column trophoblasts cultured in 1% and 5% oxygen exhibit underlying molecular differences that may contribute to the variation in column outgrowth observed in the explant cultures.
Figure 18. Differences in gene expression between 1% and 5% oxygen conditions

(A) Differentially upregulated genes in the 1% oxygen condition exclusively (53), in both the 1% and 5% conditions (239) and in the 5% oxygen condition exclusively, when compared to the 20% oxygen condition. (B) Differentially downregulated genes in the 1% oxygen condition exclusively (357), in both the 1% and 5% oxygen conditions (328) and in the 5% oxygen condition exclusively (78), when compared to the 20% oxygen condition.
Figure 19. Gene pathways upregulated in 1% and 5% oxygen conditions exclusively

(A) The top 20 pathways enriched in the 1% oxygen condition exclusively, when compared to the 20% oxygen condition. (B) The top 20 pathways enriched in the 5% oxygen condition exclusively, when compared to the 20% oxygen condition.
4.2.4 Oxygen tension regulates expression of trophoblast-specific genes

Following global gene expression analysis, I turned my attention to examining how oxygen conditions affect the expression of key trophoblast genes. I generated a curated list of 40 trophoblast-related genes from several published sources [8,49,132,138–141] (Supplemental Figure 1) and applied this list to my gene expression signature. This curated list includes genes associated with the trophoblast lineage (KRT7, TFAP2A, KLF5, GATA3), trophoblast progenitors (CDX2), CTBs (EGFR, SPINT1, ITGA6, PEG10, TEAD4), EVTs (HLA-G, FLT1, ERBB2, ADAM12, MYC, ITGA5), SCT (GCM1, CGA, ERVW-1) and genes commonly used to identify proliferative column trophoblasts and CTBs (MKI67, CCNA2). Of the 40 trophoblast specific genes, 14 were identified as being differentially expressed between the three oxygen conditions (FDR <0.05) (Figure 20). Gene cluster analyses again segregated columns into two main arms: columns cultured in one of the lower oxygen conditions (1% or 5%) and columns cultured in the 20% oxygen condition (Figure 20). Comparisons of expression of these trophoblast-specific genes across oxygen conditions identified multiple genes related to EVT differentiation and function as highly expressed within both 1% and 5% cultured columns (ADAM19, MYC, ADAM12, and ITGA5) (Figure 20). Other genes enriched within low oxygen-cultured columns include those related to trophoblast lineage (TFAP2A, GAT3, KLF5), CTB biology (EGFR, CDH1), and metalloprotease regulation (TIMP1) (Figure 20). Trophoblast-related genes shown to be enriched within 20% oxygen columns include the paternally expressed gene 10 (PEG10) and proliferation-specific genes MKI67 and CCNA2 (Figure 20). Together, these results suggest that low oxygen conditions (1% and 5%) primarily drive the expression of genes related to EVT differentiation, while the high oxygen condition (20%) drives the expression of genes associated with cell proliferation.
To more closely examine how global gene expression changes identified within low and high oxygen exposed explants relate to trophoblast differentiation, we examined the expression of the top 15 differentially upregulated genes identified in column trophoblasts cultured in 1% and 20% oxygen (FDR < 0.05; fold-change > 2) (identified through my study) in a recently reported first trimester placenta single cell transcriptomic dataset [141]. From this dataset, which identified 32 distinct cell types via uniform manifold approximation and projection (UMAP) directed clustering, we focused on 5 trophoblast subtypes within the maternal-fetal interface: CTB, proliferative CTB, SCT, proliferative EVT and EVT (Figure 21A). Expression level analysis of known trophoblast and subtype-specific genes (i.e. EGFR, ERVFRD-1, HLA-G and MKI67) confirmed the identity of these UMAP clusters (Figure 21B). A targeted heatmap analysis suggests that 1% oxygen enriched genes (Top Upregulated 1%) show expression primarily in EVT and proliferative EVT cell types. In contrast, 20% enriched genes (Top Upregulated 20%) show expression in CTB and particularly in proliferative CTB subtypes (Figure 21C). To examine how the 30 differentially upregulated genes relate to trophoblast differentiation along the villous and extravillous pathways, we utilized a pseudotime trajectory analysis. Pseudotime analysis is a computational method that allows for the identification of temporal trends in processes such as cell proliferation or differentiation from single cell genomic profiles [147]. Given that physical time cannot be captured in a snapshot of gene expression, a “pseudotime” is assigned to each cell, which provides a measure of progression or evolution in gene expression, which can then be visually represented [147]. Consistent with the results of Vento-Tormo et al., pseudotime modeling showed a specific origin of cells that likely comprise common trophoblast progenitors and two distinct trajectory arms that correspond to the villous and extravillous pathways (Figure 12D). As expected, inferred pseudotime trajectories
demonstrate a high level of expression of the syncytiotrophoblast marker ERVFRD-1 in the villous endpoint and a high level of expression of the EVT marker HLA-G in the extravillous endpoint (Figure 21E). Notably, genes identified as differentially upregulated in the 1% oxygen condition generally show a high level of expression in cells committed to the extravillous pathway (Figure 21F). Alternatively, genes identified as differentially upregulated in the 20% oxygen cultured samples are highly expressed in cells that align with progenitor and proliferative characteristics along both the villous and extravillous pathways (Figure 21F). These findings from publicly available single cell RNA sequencing data further support low oxygen as a driver of EVT differentiation and suggest that high oxygen tension may be involved in restraining EVT differentiation.
**Figure 20. Heatmap of significant trophoblast-related genes**

Heatmap of significant trophoblast related genes. This list was generated by cross-referencing the curated list of trophoblast genes with my signatures of differentially expressed genes in 1%, 5% and 20% oxygen conditions. Colour coded bars on top of the heatmap indicate experimental group (blue = 1% oxygen; purple = 5% oxygen; red = 20% oxygen) and patient ID (from black to white: P637, P638, P640, P647 and P654). Patient numbers are also listed below the heatmap with oxygen conditions shown in brackets.
Figure 21. Single cell RNA-sequencing data and oxygen condition comparisons

Previously published single cell RNA-sequencing data [141] derived from first trimester placentae (n=5) were used to investigate the cell-specificity of the oxygen-associated gene expression changes identified in my study. (A) UMAP clustering [147] of 14,366 trophoblasts extracted from the total published dataset. Cells are labelled according to their characterization by Vento-Tormo et al. [141] and colour-coded as indicated by the cell type legend: CTB, proliferative CTB (CTB p), proliferative EVT (EVT p), EVT, and SCT. (B) UMAP clustering by expression of canonical trophoblast marker genes (EGFR, ERVFRD-1, HLA-G and MKI67). (C)
Heatmap of markers for several trophoblast subtypes including pan trophoblast, general CTB, distal CCTB, proximal CCTB and syncytial pre-CTB markers along with the top 15 differentially upregulated genes in the 1% and 20% oxygen conditions, as identified from my dataset. Genes are colour-coded according to the geneset legend. (D) Pseudotime analysis of gene expression across trophoblast differentiation. (E) The inferred trajectory resulted in two distinct endpoints: cells that highly express syncytiotrophoblast marker genes (i.e. ERVFRD-1) and those that highly express extravillous trophoblast marker genes (i.e. HLA-G). (F) A heatmap of the top 15 differentially upregulated genes in the 1% and 20% oxygen conditions, constructed using inferred pseudotime. Pseudotime was ordered such that left and right represent the extravillous and villous endpoints, respectively. Hierarchical clustering segregated genes into five distinct clusters.

4.2.5 Target Genes
Unsupervised gene expression and pathway analyses revealed several genes of interest for further analysis. I decided to focus on LOX and PEG10 for their highly-ranked fold changes of 6.22 and 3.17 respectively, in comparisons of 1% and 20% oxygen conditions, along with their potential biological relevance. Based on fold change, the LOX gene was the second most significantly upregulated gene in both the 1% versus 20% and 5% versus 20% comparisons (Figure 22A). PEG10 was among the top 20 most significantly upregulated genes in the 20% versus 1% comparison and among the top 25 most significantly upregulated genes in the 20% versus 5% comparison (Figure 22B).

LOX is known to regulate covalent bonding between lysine residues of collagen and elastin fibers, contributing to the stability of the ECM [148]. In this way, it may be involved in controlling trophoblast invasion and migration through the uterine epithelium and EVT column formation [148]. PEG10 is a paternally expressed and maternally silenced gene known to play a role in cell proliferation and cancer metastasis [149]. Given the many parallels between placentation and tumorigenesis including cell invasion, vascular remodelling and immune modulation [150–153], PEG10 may provide insight into important cellular and molecular processes of placentation.

The increased expression of LOX in low oxygen observed in the microarray data was validated with a LOX activity assay. LOX activity, measured in CM generated by placental explants cultured in 1% or 20% oxygen, illustrates that LOX activity is indeed greater in 1% oxygen cultured explants when compared to 20% oxygen cultured explants (Figure 22C). Use of BAPN (competitive LOX inhibitor) demonstrates LOX-substrate specificity, while recombinant active LOX serves as a positive control (Figure 22C). Together, these results suggest that LOX expression and activity is elevated in column trophoblasts cultured in low oxygen conditions.
To garner a better understanding of the role of oxygen tension on LOX and PEG10 expression, and subsequent effects on column outgrowth, I conducted immunofluorescence microscopy on serially sectioned explants. The LOX antibody yielded a non-specific signal and thus no results could be drawn from this work (Supplemental Figure 2). Examination of PEG10 localization revealed that PEG10 is broadly localized to CTB in explants exposed to all three oxygen culture conditions. However, a bright PEG10 signal was also observed in prox CCTB of 20% oxygen cultured explants; this column-specific signal was minimal or absent in the 1% and 5% oxygen cultured explants (Figure 23).
Figure 22. LOX is upregulated in low oxygen and PEG10 is upregulated in high oxygen

(A) LOX expression levels in 1%, 5% and 20% oxygen conditions. (B) PEG10 expression levels in 1%, 5% and 20% oxygen conditions. (C) LOX activity, measured by fluorescent signal of Amplex Red in a positive control (4 µg/mL recombinant LOX), negative control (plain culture media), 1% oxygen cultured explant CM, and 20% oxygen cultured explant CM. Activity in each condition was tested with and without the addition of 5mM BAPN. Bars represent standard deviation from the mean.
Figure 23. Representative Images of PEG10 stains

PEG10 (purple) and Ck7 (green) expression in paraffin embedded placental explant cross sections cultured in 1%, 5% and 20% oxygen conditions. Nuclei are stained with DAPI (blue). Inset images in the bottom right highlight proximal column trophoblasts.
4.3 Conclusion

In this chapter, I examined global gene expression signatures to identify genes and molecular pathways affected by oxygen tension that may be involved in EVT differentiation and column formation. I identified several hundred upregulated and downregulated genes when comparing the 1% versus 20% and 5% versus 20% oxygen cultured samples and I used these signatures to further investigate the molecular pathways involved. I showed that culture in 1% and 5% oxygen led to enrichment of pathways involved in ECM structure and organization, response to steroid hormones, and growth factor and cytokine binding. Alternatively, culture in 20% oxygen led to enrichment of pathways involved in promotion of the cycle. I verified this finding with a BrdU pulse-chase experiment, which showed increased BrdU incorporation in 20% oxygen cultured columns.

Though global analyses revealed no differentially expressed genes between 1% and 5% cultured samples, comparisons of the 1% versus 20% and 5% versus 20% signatures highlight several uniquely 1%, or uniquely 5% enriched genes for further investigation. Pathway analyses of these signatures suggest that 1% oxygen enriches for pathways involved in response to reactive oxygen species, while 5% oxygen enriches for pathways involved in biosynthetic, metabolic and homeostatic processes. These findings suggest that the 5% oxygen cultured explants experience less oxidative stress and potentially healthier column development than the 1% oxygen cultured explants.

To gain better insight into the effects of oxygen tension on trophoblast differentiation, we examined the expression of trophoblast specific genes in my dataset and in a single cell RNA-sequencing dataset. Through these analyses, we show that genes enriched in 1% oxygen associate with differentiated EVTs, while genes enriched in 20% oxygen associate with
proliferative CTBs. Together with the pathway analyses, these findings promote hypoxia as a driver of differentiation down the extravillous pathway.

Finally, I identified the LOX and PEG10 genes, which were significantly upregulated in 1% and 20% oxygen conditions respectively, as potentially relevant targets for further investigation. The increased expression of the LOX and PEG10 genes observed in the 1% and 20% cultured columns via gene expression microarray were validated with the LOX activity assay and PEG10 immunofluorescence microscopy analysis.

The global gene expression, pathway analyses, targeted analyses and validation experiments discussed in this chapter led to novel insights that help explain the differences in column outgrowth and morphology observed between oxygen conditions. Furthermore, findings from this chapter help to clarify the disagreement in the literature regarding the effects of oxygen tension on trophoblast differentiation.
CHAPTER 5: THE ROLE OF LOX IN COLUMN OUTGROWTH

5.1 Introduction

Through the global gene expression analyses discussed in the previous chapter, I determined that the LOX gene was the second most significantly differentially expressed gene in low oxygen conditions. As previously mentioned, LOX is a copper-dependent monoamine oxidase known to be involved in ECM organization through its ability to catalyze covalent cross-linkages between collagen and elastin fibers [148]. LOX expression and activity are dependent on oxygen tension because molecular oxygen is required for the catalytic activity of LOX, which results in the production of hydrogen peroxide and ammonia [148]. Recall that pathway analyses in the previous chapter showed enrichment of ECM structure and organization-related processes in low oxygen conditions. Given the molecular function of the LOX gene and its regulation by oxygen tension, it may play an important role in controlling trophoblast migration and invasion during early placentation. The goal of this chapter is to functionally test the importance of the LOX gene in EVT column outgrowth.

5.2 Results

Findings from the previous chapter suggest that LOX activity is upregulated in low oxygen conditions, while its localization in prox CCTB suggests LOX likely plays a role in controlling trophoblast column outgrowth. To test the function of LOX in column outgrowth, Matrigel-embedded placental explants (n=3) were cultured in 1% oxygen in either control explant media or media containing BAPN. The effectiveness of BAPN in inhibiting LOX activity was measured as before by examining the ability of endogenous LOX in explant CM to oxidize its substrate, DAP, resulting in hydrogen peroxide production (Figure 24A). CM
harvested from control explants showed LOX activity levels slightly below those measured in recombinant LOX positive control reactions, but significantly higher than explant media alone (Figure 24A). Importantly, treatment of explants with BAPN significantly blunted LOX activity, though activity was not completely blocked (Figure 24A). However, placental explant treatment with BAPN led to a significant two-fold impairment in column outgrowth (Figure 24B, C). Taken together, these results suggest that LOX expression promotes column outgrowth and associates with an EVT phenotype.
Figure 24. Explants cultured in the presence of BAPN show decreased column area

(A) LOX activity, measured by fluorescent signal of Amplex Red, in recombinant LOX positive control, negative control, 1% oxygen cultured explants in the absence of BAPN, and 1% cultured explants in the presence of 500 μM BAPN. (B) Representative images of control and BAPN treated explants at zero and 48 hours of culture in 1% oxygen (columns traced with red dotted line). (C) Quantified column area from a validation cohort of explants (n=3) cultured in the presence or absence of 500μM BAPN for 48 hours. Control explants are shown in blue and inhibitor-treated explants are shown in red. Bars represent standard deviation from the mean.
5.3 Conclusion

In this chapter, I targeted the LOX gene to examine its importance in regulating column outgrowth. I show that BAPN effectively inhibits LOX activity and that explants cultured in the presence of BAPN for 48 hours exhibit a significant decrease in column outgrowth area when compared to explants cultured in control media. These results suggest that LOX activity is important for column outgrowth in low oxygen tension. Thus, LOX is likely involved in the pro-EVT phenotype and increased column outgrowth observed in the low oxygen cultured samples.
CHAPTER 6: OVERALL DISCUSSION

In this thesis project, I used human placental explants in culture to investigate the effects of low (1%), physiologically normal (5%) and high (20%) oxygen conditions on trophoblast differentiation and EVT column outgrowth. The purpose of this discussion is to summarize my key findings and their significance in the field of reproductive biology, address the strengths and limitations of my research, and discuss potential future directions.

6.1 Low oxygen promotes column outgrowth

The first aim of this project was to examine how low, moderate and high oxygen levels affect trophoblast column outgrowth. First, this research demonstrates that placental explants are an effective model for observing anchoring column outgrowth. My results suggest that culture in 1% or 5% oxygen led to a statistically significant increase in column outgrowth when compared to culture in 20% oxygen. This finding is both in agreement and in opposition to previous research. For example, Genbacev et al. and Caniggia et al. found that first trimester villous explants cultured in low oxygen conditions (1-3%) exhibit an increase in EVT column outgrowth [89,90]. They attribute this observation to increased proliferation through a HIF1-α directed mechanism. In direct contrast, James et al. demonstrate that villous explants cultured in 1.5% oxygen produce significantly less EVT column outgrowth when compared to those cultured in 8% oxygen [91].

Subtle differences in culture media, gestational age, matrix composition and study design may explain these opposing outcomes. For example, Caniggia et al. used 5-14 week GA samples, which are significantly older than the 5-8 week GA samples used in my experiments. Importantly, the percentage of explants resulting in column outgrowth has been shown to
decrease with increasing GA [76]. Another factor potentially contributing to the variation in published results is depth of culture media. For the purpose of cell culture experiments, most researchers control oxygen tension by adjusting the concentration within the incubator [154]. However, evidence suggests that the concentration of oxygen actually experienced by the cells decreases with increasing culture media depth [154]. Given the relatively small volume (200 μL) of culture media used in my experiments, this is unlikely to have a large effect. However, it may be important to consider when interpreting the results of other studies. Finally, details in experimental design may contribute to conflicting results. I established my explants in 5% oxygen for 24 hours and then divided them evenly between experimental conditions, based on early signs of outgrowth. This methodology helped to ensure that each condition received the same distribution of healthy explants. Other researchers who use placental explants divide their samples into experimental conditions from time zero, and thus miss the opportunity for an initial assessment of column health. Based on experience of the Beristain lab, the column outgrowth potential of a given sample or placental villi is difficult to predict by visual inspection. Without an evaluation of baseline outgrowth, it is possible that the observed effects of other investigators are a result of variation in explant health, rather than oxygen condition. In light of the conflicting literature, the results from aim 1 contribute to a growing body of evidence in support of low oxygen as a driver of increased column outgrowth.

Complementary to my objective measurements, I observed clear differences in column morphology between conditions. Specifically, the 1% oxygen cultures showed elongated spikes of outgrowth in the distal column and the 20% oxygen cultures showed dense plumes of outgrowth in the proximal column. To my knowledge, such detailed observations of the differences in EVT column morphology have not been previously described. These observations
provide preliminary evidence that oxygen tension is involved in controlling trophoblast migration and proliferation.

6.2 Low oxygen drives EVT differentiation and high oxygen drives CCTB proliferation

The second aim of this project was to identify specific molecular functions and gene pathways affected by the three oxygen conditions of interest (1%, 5% and 20%). Global gene expression signatures identified several hundred upregulated and downregulated genes in comparisons between the two lower oxygen conditions (1% and 5%) and the high oxygen condition (20%). The low oxygen conditions showed enrichment of biological pathways largely involved in response to hypoxia (HIF-1 signalling), ECM structure and organization, response to steroids, and metabolism. These pathways likely drive column outgrowth by promoting trophoblast invasion through ECM degradation, and migration through increased cellular metabolism and motility. The high oxygen condition showed enrichment of pathways involved in promotion of the cell cycle, which suggests that high oxygen promotes column outgrowth through a proliferative phenotype. I confirmed this observation with a pulse-chase experiment, which showed an increase in BrdU incorporation in the 20% cultured columns when compared to the 1% and 5% cultured columns. Furthermore, through examination of single cell RNA-sequencing data, I show that low oxygen promotes expression of EVT genes such as ITGA5, ADAM12 and FLT1 and transcription factors like KLF5 and GATA3 that are highly expressed in EVT subtypes. Alternatively, high oxygen promotes the expression of genes primarily expressed in proliferative CTB populations. Together, this data suggests that low oxygen drives column trophoblast differentiation towards an EVT-like phenotype and that high oxygen
promotes trophoblast proliferation. These findings are both supported and opposed in the literature.

For example, research by James et al. supports my findings. They suggest that exposure to hypoxia in the first trimester of pregnancy results in changes in gene expression including an increase in transcription factor, hypoxia-related (HIF) pathways and metabolism-related pathways [76]. Additionally, Graham et al. show that HTR-8 cells cultured in 1% oxygen show greater invasion through Matrigel matrix when compared to those cultured in 20% oxygen [155]. Nevertheless, my results are in direct contrast to the findings of other groups. In another study by Genbacev et al. looking specifically at trophoblast invasion and PE, lowering oxygen conditions from 20% to 2% led to a decrease in invasion through ECM [156]. Furthermore, research by both Genbacev et al. and Jiang et al. showed that culture of trophoblasts in hypoxic conditions led to a significant increase in BrdU incorporation [156,157]. Finally, other studies have shown that HTR-8 cells cultured in hypoxic conditions demonstrate an increase in proliferation [158,159] and reduced invasion into Matrigel matrix [158]. However, much of this research was conducted more than 20 years ago using a variety of models including primary and immortalized cell lines.

My study offers the 3D and multicellular advantages of human placenta explants in culture, combined with high dimensional gene expression data, and thus provides a more holistic view of the effects of oxygen tension on trophoblast differentiation. Along with the results from explant outgrowth experiments, the gene expression analyses suggest that low oxygen promotes increased column outgrowth and trophoblast differentiation along the extravillous pathway. In this way, my research may help to resolve the ongoing disagreement about the effects of oxygen tension on trophoblast biology and anchoring column outgrowth.
6.3 The role of LOX in EVT differentiation

The third aim of my project was to functionally test the importance of novel genes or gene pathways in trophoblast column outgrowth. I focused on the LOX gene for its high log fold change in the 1% oxygen cultures and its potential biological relevance to column outgrowth. The LOX gene encodes for a family of copper-dependent amine oxidases, which are known to regulate formation and repair of the ECM by catalyzing covalent cross linkages between collagen and elastin fibres [148,160,161]. LOX family oxidases promote cancer metastasis and are upregulated in tumour epithelial cells [160,161]. Importantly, hypoxia is known to promote tumour angiogenesis, invasion and metastasis via HIF-1 signalling [161]. LOX expression is similarly controlled by HIF-1 and VEGF [160]. Given the highly invasive nature of human trophoblasts, several hypotheses have been proposed regarding a shared evolutionary history between cancer metastasis and placentation [162]. The LOX gene has been studied extensively in cancer biology, but minimally in the context of placental development. However, LOX family oxidases have been shown to be highly expressed in several tissues of the reproductive system [163] and to be involved in the strength of the amniotic sac with implications for preterm rupture of fetal membranes [164,165]. Given these functional characteristics of the LOX gene and its significant upregulation in the low oxygen cultured samples, it was an ideal candidate for further investigation.

To examine the importance of the LOX gene in EVT column outgrowth, I cultured explants in 1% oxygen in the presence of BAPN. Explants cultured in BAPN showed significantly reduced column area when compared to control explants, suggesting that LOX plays a critical role in column outgrowth. LOX is thought to regulate invasion through focal adhesion kinase activity and cell-ECM adhesion [166]. Therefore, I propose that LOX expression
and subsequent collagen crosslinking promotes column outgrowth by providing a substratum scaffolding on which EVTs can more readily migrate.

Importantly, there are five members of the LOX family including LOX and LOX-like gene 1-4 [167,168]. Based on variation in the C-terminal domains of the LOX family genes, they may serve biological functions outside of their catalytic role, but these are unknown [167]. LOX expression is regulated by oxygen through a HIF-dependent pathway [166]. This mechanistic understanding is echoed in my data, which shows increased expression of hypoxia and HIF-related pathways, along with increased LOX expression in the 1% enriched samples. In addition to the catalytic function of LOX, there is evidence to suggest that it may be involved in gene expression regulation, cell growth, adhesion and migration [168]. Therefore, the role of LOX in promoting column outgrowth may not be limited to cell-ECM interactions. Other mechanisms more directly related to trophoblast differentiation may be involved, but identification of these mechanisms would require further investigation.

Finally, consistent with my results, knockdown of LOX or LOXL2 has been shown to cause a reduction in trophoblast migration and invasion [169]. Recently, decreased LOX expression has been observed in placentae from preeclamptic pregnancies [170]. The authors of this research propose collagen accumulation caused by the downregulation of LOX and LOXL2 as a potential mechanism for the impaired trophoblast migration and invasion observed in PE [170]. Therefore, LOX may be involved in the etiology of pregnancy complications such as PE and IUGR.

My findings are consistent with the current knowledge of the function and regulation of the LOX gene and provide novel insights into the potential role of LOX in regulating EVT invasion and column outgrowth.
6.4 The role of PEG10 in CCTB proliferation

To further investigate the effect of hyperoxia on column outgrowth, I decided to focus on PEG10 given its high log fold change in the 20% oxygen cultures and potential biological relevance. PEG10 is a maternally imprinted gene localized on human chromosome 7 that is known to be important in placenta development [171]. For example, silencing of PEG10 in human placental explants results in impaired villous trophoblast function [172]. Moreover, pregnant Peg10 knockout mice undergo spontaneous abortion at 10 days GA due to failed placentation [149]. However, the exact role of PEG10 in placentation is unknown. Evidence suggests that PEG10 is involved in inhibiting apoptosis and encouraging cell proliferation [149]. It is also thought to promote the proliferation of several carcinomas, a finding that has been validated through knockdown experiments [173]. Previous histological examination of primary placental tissue show that PEG10 is expressed in the SCT, CTB, endothelial cells and stroma [174]. These findings are consistent with my data, which suggests that PEG10 is highly expressed in the CTB, particularly in proliferative CTB populations. Furthermore, I observed significant upregulation of PEG10, along with a strong proliferative phenotype based on differential gene expression analysis and visual evidence of proliferation in the 20% cultured explants. A recent study by Skiles et al. demonstrates that culture of mouse embryonic stem cells in atmospheric oxygen causes dysregulation of chromatin modifying genes and loss of genomic imprinting [175]. Thus, the increase in PEG10 expression observed in the 20% oxygen culture condition may be explained through a general loss of imprinting. Finally, PEG10 is known to be overexpressed at both the mRNA and protein levels in preeclamptic placentae [174]. Recall that PE is associated with poor EVT invasion and spiral artery remodelling [64]. Given the highly proliferative phenotype in the 20% cultured explants, in contrast to the invasive phenotype of the
1% and 5% cultured explants, the upregulation of PEG10 that I observed may be associated with the impaired EVT differentiation and trophoblast invasion characteristic of PE. In this way, my research may provide some preliminary insight into the relationship between PEG10 expression and important placental pathologies.

6.5 Experimental strengths and limitations

Like all research projects, this thesis has both unique strengths and weaknesses. The major strength of this project is the choice of model and experimental design. The use of human placental explants in culture is a strength for several reasons. First of all, a human model is superior to an animal model, particularly for the study of key trophoblast behaviours such as invasion, migration and differentiation because few animals share equivalent placental morphology and depth of trophoblast invasion with humans. The major benefit of animal models is their organism-level complexity. The explant model offers some of this benefit through its 3D structure and incorporation of multiple cell types. The explant model shows more morphological similarity to placental villi in-vivo than primary cultures or cell lines. Given its human origin, structural integrity and multicellular complexity, the human placental explant in culture is arguable one of the best models for the study of trophoblast biology and human placentation.

The second aim of my project combines the use of an explant model with high-throughput, transcriptome-wide microarray data. Specifically, I extracted RNA from the column cells and EVTs that had invaded into the Matrigel matrix and subjected them to a gene expression analysis. This component of the project design provided invaluable insight into the molecular and cellular processes underlying the explant outgrowth results. I used a microarray instead of newer methods such as single cell RNA-sequencing for cost effectiveness and
confidence in the well-established pipelines and statistical methodologies. My analyses led to novel results that will help to clarify the highly discordant existing beliefs regarding the effects of oxygen on placenta development. To my knowledge, this project is the first to combine human placental explants and microarray data on invasive column cells specifically, in a study of oxygen tension.

Finally, I made a concerted effort to validate my results through several methods. I conducted a pulse-chase experiment with BrdU incorporation, which supported the proliferative phenotype observed in the 20% oxygen cultured explants. I conducted immunofluorescence microscopy with LOX and PEG10 antibodies to validate the localization and expression of these target genes in explant columns. Finally, I conducted LOX activity and inhibition assays to further interrogate the importance of this gene in column outgrowth.

Despite the varied strengths of this research, there are also some limitations to consider. For example, I exposed the explants to 1%, 5% or 20% oxygen consistently for 48 hours. This is not representative of the in-vivo condition, where placental villi are exposed to a gradient of oxygen. In-vivo, the villi that have invaded deeper into the endometrium will be exposed to higher oxygen conditions than those with shallower invasion. Villi in the center of the discoid placenta would also be exposed to greater oxygen levels than those on the periphery. As maternal blood is delivered in spurts and pools, oxygen exposure of chorionic villi is in constant fluctuation. Furthermore, the explant model fails to capture several aspects of the in-vivo condition. For example, while the Matrigel protein matrix recapitulates the ECM of the uterine stroma, it does not model the uterine epithelial layer, through which the EVT column must penetrate. Uterine glands, along with both maternal and fetal circulations are also missing from the model. Another potential weakness of the experimental design is that explants are
challenging to work with. Their ability to proliferate and invade a basement membrane is unpredictable, it is challenging to manipulate their gene expression, and they are difficult to examine histologically due to their delicate structure. My attempts to examine LOX expression through immunohistochemistry were unsuccessful due to a non-specific signal. Additionally, I opted to use a microarray for gene expression analysis for cost efficiency and ease of use. However, microarray analyses do not allow for identification of micro-RNAs or non-coding RNAs like bulk-sequencing, which may have provided more comprehensive insight into explant transcriptomes. I targeted the LOX gene with a pharmacological inhibitor and examined changes in column outgrowth. Given the function of LOX, I suspect the observed outcome is a result of changes in the ability of EVTs to invade the Matrigel matrix. However, I cannot be certain of the specificity of BAPN. BAPN is known to functionally inhibit other lysyl oxidase-like genes including LOXL2 [176], which was significantly upregulated in my 1% oxygen cultures. Therefore, it is likely inhibiting outgrowth through its interaction with several genes at varying degrees of specificity. Finally, it may also have toxic effects on cells, thus contributing to other off-target effects. Further investigations would have to be conducted to confirm the specificity of this reagent and improve confidence in the importance of the LOX gene in EVT column outgrowth. Finally, only five biologically distinct placentae were used in the gene expression microarray. This small sample size may explain why no differentially expressed genes were identified in comparisons of the 1% and 5% oxygen cultured samples using an FDR < 0.05.
6.6 Future directions

Given more time and resources, there are a number of future directions that this project could take. First of all, it would be very interesting to conduct single-cell RNA sequencing on column and EVT cells extracted from the Matrigel of placenta explants cultured in various oxygen conditions. This experimental design would provide significant insight into the biological mechanisms controlling column outgrowth and allow the investigator to attribute function to specific column cell types, which has previously been a weakness of placental explants and other culture methods. As part of my graduate research, I have been involved in preliminary 10X Genomics single-cell RNA sequencing experiments on primary isolated CTBs. The Beristain lab has an incoming PhD candidate who will take over the explant work and incorporate single-cell methods. I attempted to mechanistically interrogate the LOX gene to determine its importance in column outgrowth, but I did not conduct a parallel experiment with the PEG10 gene. Further investigation should focus on testing the functional significance of PEG10 in column outgrowth. Moreover, based on the results of the gene expression analysis, there are other target genes that should be further investigated. For example, TNFSF10 was the most differentially upregulated gene in the 1% oxygen condition when compared to the 20% oxygen condition, with LOX being the second most differentially expressed gene in this signature. Given limited time and resources, I chose to investigate LOX for its known biological relevance in ECM interactions and metastasis. With more time and resources, future efforts should focus on functional investigations of the TNFSF10 gene. Furthermore, several gene expression studies have been conducted with a focus on dysregulation of gene expression in pregnancies affected by PE [177,178]. Specifically, research has identified the FMS-like tyrosine kinase 1 (FLT1) as a potential driver for the genetic basis of PE. Soluble FLT1 encodes a splice variant of the VEGF
receptor and is this involved in angiogenesis. Excess circulation of soluble FLT1 leads to the hypertension and proteinuria observed in PE [179]. Interestingly, FLT1 was significantly upregulated in my 1% oxygen cultured explants. Therefore, it may be involved in mediating an association between oxygen dysregulation in the first trimester of pregnancy and the pathophysiology of PE. Future work should be done to synthesize the gene expression results from my research with the results of studies examining gene expression in pregnancy disorders such as PE and IUGR. Finally, I showed that inhibition of the LOX gene decreases column outgrowth, but the question remains whether its function is limited to ECM interactions, or whether it plays a more involved role in EVT differentiation and migration. Future research should focus on investigating this question more completely.

6.7 Conclusion

Oxygen tension is one of several factors controlling early trophoblast differentiation and anchoring column formation. Column outgrowth occurs in low, moderate and high oxygen conditions, but is significantly increased in hypoxic conditions. Low oxygen appears to drive column outgrowth through a migratory and invasive phenotype while high oxygen appears to drive column outgrowth through increased proliferation. The PEG10 gene is highly expressed in several placental cell types and is significantly upregulated in 20% oxygen, potentially contributing to the increase in proliferation observed in that condition. Expression of the LOX gene is significantly upregulated in 1% oxygen and appears to be a requirement of column outgrowth, likely through its catalytic function in collagen crosslinking. My graduate research provides novel insights and helps to clarify significant disagreement in the literature regarding
the effects of oxygen tension on trophoblast differentiation and healthy placentation during the first trimester of pregnancy.
References


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Supplemental Figure 1. Curated list of epithelium and trophoblast specific genes

(A) Trophoblast specific genes appearing in this figure were curated from several sources [49,85,132,138–141]. (B) Epithelium specific signature was generated from the ToppGenes GO term “epithelium development” (GO:0060429).
Supplemental Figure 2. Representative Images of LOX stains

LOX (green) and Ck7 (pink) localization in column trophoblasts cultured in 1%, 5% and 20% oxygen conditions. DAPI (blue) represents cell nuclei. Inset images represent LOX (white) only.